ISSN 2186-3644 Online ISSN 2186-361X

IRDR

Intractable & Rare Diseases Research

Volume 12, Number 3
August, 2023

www.irdrjournal.com

IRDR
Intractable & Rare Diseases Research

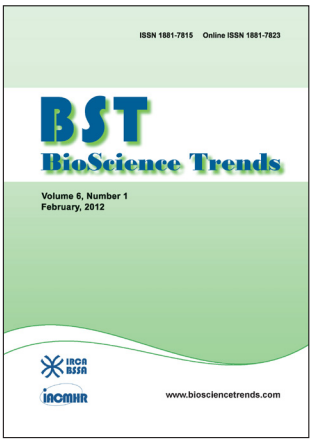
ISSN: 2186-3644
Online ISSN: 2186-361X
CODEN: IRDRA3
Issues/Year: 4
Language: English
Publisher: IACMHR Co., Ltd.

Intractable & Rare Diseases Research is one of a series of peer-reviewed journals of the International Research and Cooperation Association for Bio & Socio-Sciences Advancement (IRCA-BSSA) Group and is published quarterly by the International Advancement Center for Medicine & Health Research Co., Ltd. (IACMHR Co., Ltd.) and supported by the IRCA-BSSA.

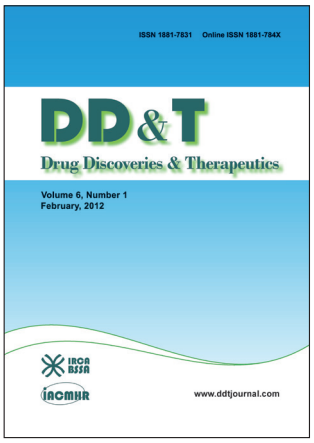
Intractable & Rare Diseases Research devotes to publishing the latest and most significant research in intractable and rare diseases. Articles cover all aspects of intractable and rare diseases research such as molecular biology, genetics, clinical diagnosis, prevention and treatment, epidemiology, health economics, health management, medical care system, and social science in order to encourage cooperation and exchange among scientists and clinical researchers.

Intractable & Rare Diseases Research publishes Original Articles, Brief Reports, Reviews, Policy Forum articles, Case Reports, Communications, Editorials, News, and Letters on all aspects of the field of intractable and rare diseases research. All contributions should seek to promote international collaboration.

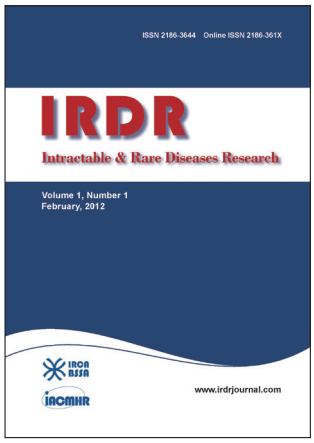
IRCA-BSSA Group Journals



ISSN: 1881-7815
Online ISSN: 1881-7823
CODEN: BTIRCZ
Issues/Year: 6
Language: English
Publisher: IACMHR Co., Ltd.
www.biosciencetrends.com



ISSN: 1881-7831
Online ISSN: 1881-784X
CODEN: DDTRBX
Issues/Year: 6
Language: English
Publisher: IACMHR Co., Ltd.
www.ddtjournal.com



ISSN: 2186-3644
Online ISSN: 2186-361X
CODEN: IRDRA3
Issues/Year: 4
Language: English
Publisher: IACMHR Co., Ltd.
www.irdrjournal.com

Intractable & Rare Diseases Research

Editorial and Head Office

Pearl City Koishikawa 603, 2-4-5 Kasuga, Bunkyo-ku,
Tokyo 112-0003, Japan

E-mail: office@irdrjournal.com
URL: www.irdrjournal.com

Editorial Board

CONTENTS

Volume 12, Number 3, 2023

Policy Forum

Review

Original Article

Brief Report

Correspondence

Letter

Policy Forum

DOI: 10.5582/irdr.2023.01030

Analysis of marketed orphan drugs in China

Wei Zhi, Meilin Liu, Dan Yang, Shanshan Zhang, Yanqin Lu*, Jinxiang Han*

Key Laboratory for Biotech Drugs of the National Health Commission, Key Laboratory for Rare & Uncommon Diseases of Shandong Province, Biomedical Sciences College & Shandong Medicinal Biotechnology Centre, Shandong First Medical University & Shandong Academy of Medical Sciences, Ji'nan, Shandong, China.

SUMMARY In recent years, China has increased attention on the issue of rare diseases, and the government has promulgated rare disease–related policies to gradually improve rare disease diagnosis, treatment, drug marketing, and patient burden. Orphan drugs were added to the medical insurance directory in 7 batches, of which 22 drugs were first included in the 2004 medical insurance directory and 8, 16, 12, 7, 8, and 7 were included in the 2009, 2017, 2019, 2020, 2021, and 2022 versions, respectively. Currently, 106 orphan drugs are marketed in China, which are suitable for treating 53 rare diseases such as hematologic diseases, congenital metabolism disorders, neuropathies, and digestive system diseases and for other treatment fields. The drugs are mainly manufactured in 15 countries such as China, Switzerland, and the USA, of which 10 drugs can be used to treat different rare diseases. At the same time, there are multiple treatments available for 25 rare diseases. In this paper, we examined the manufacturers, marketing status, indications, and inclusion of orphan drugs in the National Basic Medical Insurance Directory to describe and analyze the current status of 106 orphan drugs that are currently marketed in China to provide a reference for rare disease policy formulation and drug development.

Keywords marketed orphan drugs, rare diseases, medical insurance, indications for medication

1. Introduction

Rare diseases refer to rarely encountered diseases with low prevalence and are also known as orphan diseases. The World Health Organization defines rare diseases as diseases with an incidence of 0.65‰–1‰ (*1*). Currently, 6,000–8,000 unique rare diseases have been identified, of which 80% are genetic in origin and 50% occur in children (*2*). Rare diseases are often chronic and progressive diseases that have significant incidence and mortality rates (*3*). The issue of orphan drugs was first mentioned by rare disease groups in the USA. In other countries, rare disease security policies mainly include formulating marketing laws for orphan drug marketing, encouraging the research and development of orphan drugs, and accelerating the marketing review and approval of orphan drugs, such as the Orphan Drug Act passed in the USA in 1983 (*4*) and the Orphan Drug Regulation promulgated and implemented in the European Union in which orphan drugs recognized in the European Union can enjoy scientific recommendations, expedited review and approval, and 10-year postmarketing market monopoly by the European Medicines Agency (*5*).

In recent years, China has focused increasing attention on rare disease medical insurance, and rare disease medical insurance policies in China have been formulated from scratch. On the one hand, the Chinese government has promulgated a series of policies to encourage the development and marketing of orphan drugs, which has achieved some progress in increasing the number of orphan drugs. These policies include supporting companies in research and development of orphan drugs and encouraging the exploration of new rare disease indications for marketed drugs in the area of encouraging the development of orphan drugs (*6*) and providing expedited review and approval for orphan drugs in the area of priority review and approval (*7*) and corresponding policies for clinical trial exemption and tax reduction. On the other hand, as rare diseases are prone to misdiagnosis and missed diagnosis, a national diagnosis and treatment cooperative network was established by China's healthcare system to construct a rare disease case information registration and management system. In October 2019, the National Health Commission promulgated the China Rare Disease Diagnosis and Treatment Service Information System Working Management Protocol requiring

member hospitals in the cooperative network to perform diagnosis and treatment information registration for rare disease cases and set up an information system to collect relevant data (*8*). In addition, local governments have actively explored rare disease administration security mechanisms. After many years of practice, local rare disease administration security mechanisms have gradually become the "project fund" model represented by Zhejiang and Jiangsu (*9*), the "major disease insurance" model represented by Shandong and Chengdu (*10*), and "medical relief" represented by Foshan (*11*). Overall, the rare disease security attempts and practices of some provinces and cities resulted in the "expansion from a point" development of rare disease policies and healthcare security and accumulated practice experience for rare disease security policies at the national level.

The *Notice on First Batch of Rare Disease Directory* released in May 2018 included a total of 121 rare diseases (*12*). The 2020 Opinions on *Deepening the Reform of the Medical Security System* by the State Council of the People's Republic of China explored rare disease administration security mechanisms, strengthening basic medical insurance, major disease insurance, and medical relief security systems, and encouraged the development of commercial health insurance and social charity relief (*13*). In June 2022, the National Healthcare Security Administration released the 2022 National Basic Medical Insurance, Work-Related Injury Insurance, and Maternity Insurance Drug Directory Adjustment Working Plan. The plan clearly stated prioritizing rare disease patients, removed application time limits for orphan drugs, and supported inclusion into the national generic drug directory at the same time (*14*). On January 18, 2023, the National Healthcare Security Administration printed the National Basic Medical Insurance, Work-Related Injury Insurance, and Maternity Insurance Drug Directory (2022) notice and encouraged active exploration of using "dual-channel" to improve the supply and security levels of orphan drugs (*15*).

2. Existing orphan drugs and drug manufacturers

In recent years, the National Healthcare Security Administration has exerted efforts in improving the affordability of orphan drugs. Since 2017, when the National Health Insurance Directory was routinely adjusted and examined, the National Healthcare Security Administration has appropriately aimed at orphan drug declaration and attention. After enquiring the National Healthcare Security Administration and searching the Wuwang Database and Yaozhi Database, the number of approved drugs for treating rare diseases in China (Table 1) was calculated. It was found that 106 drugs could be used to treat 53 rare diseases, such as hematologic diseases, congenital metabolism disorders, neuropathies, and digestive system diseases. From Table 1, it can be observed that 83 orphan drugs were included in the National Health Insurance Directory as of March 2023, of which 22 drugs were first included in the 2004 medical insurance directory and 8, 16, 12, 7, 8, and 7 were included in the 2009, 2017, 2019, 2020, 2021, and 2022 versions, respectively. These 80 drugs cover a total of 40 rare diseases, of which 13 are under class A reimbursement in the National Health Insurance Directory and used to treat 10 rare disease indications without requiring patient self-pay and 67 drugs are under class B reimbursement, which requires patients to pay a specific proportion of the drug fees on the basis of the provincial medical insurance policy and in which part of the cost is reimbursed. The reimbursement ratio differs according to local policies and drugs.

There are 106 orphan drugs, and all are Western medicines. From Figure 1, it can be observed that manufacturers are located in 15 countries, of which 26 drugs were developed by 23 Chinese companies such as Zhaoke Pharmaceutical Co. Ltd. and Shandong Taibang Biological Products Co. Ltd. The remaining 67 drugs are manufactured in 14 other countries, mainly Switzerland, the USA, and Germany. From the statistical analysis in Table 1, it can be observed that Novartis AG from Switzerland developed 11 drugs, which are used to treat six rare diseases. Among them, four are used to treat multiple sclerosis. Furthermore, Actelion from Switzerland developed three drugs for idiopathic pulmonary hypertension. In the USA, the main pharmaceutical companies that developed drugs for rare diseases are Merck Sharp & Dohme, Biogen, and Pfizer. There are 11 drugs that were developed in Germany, of which six were developed by Bayer. Sanofi in France developed seven drugs, Takeda in Japan developed five drugs, and AstraZeneca and GSK in the UK developed six drugs.

3. Current status of marketed orphan drugs in China

After searching the Orphanet database, it was found that 73.5% of these 53 rare diseases are inherited, such as idiopathic pulmonary hypertension and spinal muscular atrophy. These rare diseases include immune disorders, hematologic diseases, tumors, congenital metabolic disorders, nervous system diseases, respiratory diseases, and endocrine disorders. At present, 42.5% of the drugs can be used to treat 21 different neurological disorders such as Parkinson's disease (young-onset, early onset) and multiple sclerosis, and 24% of the drugs can be used to treat 19 congenital metabolic disorders such as primary carnitine deficiency and Fabry disease (Supplementary Table 1).

From Table 2, it can be observed that there are many drugs for 25 rare diseases and that some drugs have the same mechanisms of action for treating

Table 1. The list of 106 marketed drugs approved in China for the treatment of rare diseases

Rare Disease	Drug	R&D Country	R&D Manufacturer	Time to market (abroad)	Time to market (China)	Medical insurance type (insurance period)
21-Hydroxylase Deficiency	Hydrocortisone	China	Henan Lihua Pharmaceutical Ltd	N.A.*	2001	Class-A (2004)
	Hydrocortisone Acetate	China	Meyer Pharmaceuticals Ltd	N.A.	1979	-**
Amyotrophic Lateral Sclerosis	Riluzole Oral Suspension	China	Zhaoke Pharmaceutical (Hefei) Ltd	2022	2022	Class-B (2022)
	Edaravone Injection	China	Jiangsu Zheng Dafeng hai pharmacy Ltd	N.A.	2019	Class-B (2020)
	Riluzole Tablets	France	Sanofi	1996	1998	Class-B (2017)
Atypical Hemolytic Uremic Syndrome	Eculizumab Injection	Britain	AstraZeneca Pharmaceutical Ltd	2007	2018	-
Beta-ketothiolase Deficiency	Levocarnitine	Italy	AlfasigmaS.p.A.	1990	1999	Class-B (2009)
Biotinidase Deficiency	Compound Vitamin B Tablets	Germany	Bayer Schering Pharma AG	1986	1999	Class-B (2004)
Carnitine Deficiency	Levocarnitine	Italy	AlfasigmaS.p.A.	1990	1999	Class-B (2009)
Castleman Disease	Siltuximab for Injection	America	Johnson&Johnson Pharmaceuticals Ltd	2004	2021	-
Citrullinemia	Sodium Phenylbutyrate Powder	China	Zhaoke Pharmaceutical (Guangzhou) Ltd	1996	2021	-
Congenital Adrenal Hypoplasia	Hydrocortisone	China	Henan Lihua Pharmaceutical Ltd	N.A.	2001	Class-A (2004)
	Hydrocortisone Acetate	China	Meyer Pharmaceuticals Ltd	N.A.	1979	-
Fabry Disease	Agalsidase Alfa Concentrated Solution for Infusion	Japan	Takeda Pharmaceutical Ltd	2001	2021	Class-B (2021)
	Agalsidase Beta for Injection	France	Sanofi	2001	2018	-
Gaucher's Disease	Velaglucerase Alfa for Injection	Japan	Takeda Pharmaceutical Ltd	2014	2021	-
	Imiglucerase for Injection	France	Sanofi	1994	2008	-
Generalized Myasthenia Gravis	Pyridostigmine Bromide Tablets	China	Shanghai Shangyao Traditional Chinese and Western Pharmaceutical Ltd	1955	2003	Class-A (2004) -
Glycogen Storage Disease (Type I, II)	Alglucosidase Alfa for Injection	France	Sanofi	2006	2015	-
Hemophilia	Human Coagulation Factor IX	China	China Biologic Products Holdings, Inc	N.A.	2020	Class-B (2021)
	Desmopressin Acetate	Netherlands	Ferring Pharmaceuticals Ltd	1972	2001	Class-A (2004)
	Human Prothrombin Complex	China	Shanghai Xinxing Medicine Ltd	N.A.	2002	Class-B (2004)
	Human Coagulation Factor VIII	China	Tonrol Bio-pharmaceutical Ltd	N.A.	2018	Class-A (2017)
	Recombinant Human Coagulation Factor VIII	Germany	Bayer Schering Pharma AG	1992	2007	Class-B (2009)
	Recombinant Human Coagulation factor IX	America	Pfizer Inc	1977	2012	Class-B (2017)
	Recombinant Human Coagulation Factor VIIa	Denmark	Novo Nordisk Biotechnology Ltd	1999	2002	Class-B (2017)
	Eftrenonacog alfa for Injection	France	Sanofi	2016	2021	-
	Emicizumab Injection	Switzerland	Roche Pharmaceutical Ltd	2017	2018	-
Hepatolenticular Degeneration	Penicillamine Tablets	China	Hainan Honghui Pharmaceutical Ltd	N.A.	1999	Class-A (2004)
	Sodium Dimercaptosuccinate	China	Shanghai Fudan Fuhua Pharmaceutical Ltd	N.A.	2002	Class-A (2004)
	Dimercaptosuccinic Acid	America	Recordati Rare Diseases Inc.	1991	1999	Class-A (2004)
	Zinc Sulfate	China	Shanghai Pharmaceutical Xinyi Pharmaceutical Ltd	1970	2004	Class-B (2009)
Hereditary Angioedema	Danazol Capsules	China	Baiyunshan Zhengqing Pharmaceutical Ltd	N.A.	1999	Class-B (2004)
	Icatibant Acetate Injection	Japan	Takeda Pharmaceutical Ltd	2011	2021	Class-B (2021)
	Lanadelumab Injection	Japan	Takeda Pharmaceutical Ltd	2018	2020	Class-B (2022)
Hereditary Hypomagnesemia	Magnesium Gluconate	China	Beijing Jialin Pharmaceutical Ltd	N.A.	2020	-

Table 1. The list of 106 marketed drugs approved in China for the treatment of rare diseases (continued)

Rare Disease	Drug	R&D Country	R&D Manufacturer	Time to market (abroad)	Time to market (China)	Medical insurance type (insurance period)
	Potassium Aspartate and Magnesium Aspartate	Hungary	Gedeon Richter Plc	1962	1989	Class-B (2004)
Homozygous Hypercholesterolemia	Evolocumab Injection	America	Amgen Inc.	2015	2018	Class-B (2021)
	Rosuvastatin Calcium Tablets	Britain	AstraZeneca Pharmaceutical Ltd	2002	2006	Class-B (2009)
	Ezetimibe Tablets	America	Merck Sharp & Dohme	2002	2012	Class-B (2017)
Huntington Disease	Deutetrabenazine Tablets	Israel	Teva Pharmaceutical Industries Ltd	2017	2020	Class-B (2020)
	Tetrabenazine	France	Recipharm Fontaine SAS	2008	2021	-
Hyperphenylalaninemia	Sapropterin Dihydrochloride Tablets	Germany	Excella GmbH & Co.KG	2007	2010	-
Hypophosphatemic Rickets	Burosumab Injection	Japan	Kyowa Kirin Inc.	2019	2021	-
Idiopathic Cardiomyopathy	Tafamidis Meglumine	America	Pfizer Inc	2019	2020	Class-B (2021)
Idiopathic Hypogonadotropic	Gonadorelin for Injection	China	Maanshan Fengyuan Pharmaceutical Ltd	N.A.	1996	Class-B (2004)
Hypogonadism	Menotrophins for Injection	China	Maanshan Fengyuan Pharmaceutical Ltd	N.A.	2010	Class-B (2004)
	Chorionic Gonadotrophin for Injection	China	Penglai Huatai Pharmaceutical Ltd	N.A.	2001	Class-A (2004)
Idiopathic Pulmonary Arterial	Treprostinil Injection	China	Zhaoke Pharmaceutical (Hefei) Ltd	2005	2013	Class-B (2022)
Hypertension	Ambrisentan tablets	Britain	GlaxoSmithKline	2007	2010	Class-B (2020)
	Bosentan Tablets	Switzerland	Actelion Pharmaceuticals Ltd	2001	2006	Class-B (2019)
	Riociguat Tablets	Germany	Bayer Schering Pharma AG	2013	2017	Class-B (2019)
	Macitentan Tablets	Switzerland	Actelion Pharmaceuticals Ltd	2013	2017	Class-B (2019)
	Selexipag Tablets	Switzerland	Actelion Pharmaceuticals Ltd	2018	2018	Class-B (2019)
	Sildenafil Citrate Tablets	America	Pfizer Inc	1998	2000	-
	Iloprost	Germany	Bayer Schering Pharma AG	2003	2018	-
Kallmann Syndrome	Gonadorelin for Injection	China	Maanshan Fengyuan Pharmaceutical Ltd	N.A.	1996	Class-B (2004)
	Menotrophins for Injection	China	Maanshan Fengyuan Pharmaceutical Ltd	N.A.	2010	Class-B (2004)
	Chorionic Gonadotrophin for Injection	China	Penglai Huatai Pharmaceutical Ltd	N.A.	2001	Class-A (2004)
Idiopathic Pulmonary Fibrosis	Nintedanib esilate soft capsules	Germany	Boehringer Ingelheim Pharma GmbH & Co. KG	2014	2019	Class-B(2020)
	Pirfenidone Capsules	China	Beijing Cantiny Pharmaceutical Ltd	2008	2013	Class-B(2017)
Inborn Errors of Bile Acid Synthesis	Sodium Cholate Tablets	China		N.A.	2002	-
Mucopolysaccharido-sis	Laronidase concentrated solution for infusion	France	Changzhou Qianhong Biochemical Pharmaceutical Ltd Sanofi	2003	2020	-
	Elosulfase alfa injection	Germany	Vetter Pharma-Fertigung GmbH & Co.KG	2014	2019	-
	Idursulfase beta Injection	Korea	Green Cross Corporation	2012	2020	-
Multiple Sclerosis	Fampridine	America	Biogen, Inc.	2010	2021	Class-B (2021)
	Fingolimod Hydrochloride Capsules	Switzerland	Novartis Pharma Schweiz AG	2010	2019	Class-B (2020)
	Siponimod Tablets	Switzerland	Novartis Pharma Schweiz AG	2019	2020	Class-B (2020)
	Teriflunomide Tablets	France	Sanofi	2012	2018	Class-B (2019)
	Baclofen Tablets	Switzerland	Novartis Pharma Schweiz AG	1977	1994	Class-B (2004)
	Dimethyl Fumarate Enteric Capsules	America	Biogen, Inc.	2013	2021	Class-B (2022)
	Ofatumumab Injection	Switzerland	Novartis Pharma Schweiz AG	2020	2021	Class-B (2022)
	Interferon beta-1b	Germany	Bayer Schering Pharma AG	1993	2018	-
	Recombinant human interferon beta-1b for injection	Germany	Bayer Schering Pharma AG	1993	2018	-

Table 1. The list of 106 marketed drugs approved in China for the treatment of rare diseases (continued)

Rare Disease	Drug	R&D Country	R&D Manufacturer	Time to market (abroad)	Time to market (China)	Medical insurance type (insurance period)
Multiple System Atrophy	Droxidopa Capsules	China	Chongqing Shenghuaxi Pharmaceutical Ltd	1989	2012	Class-B (2017)
Neonatal Diabetes Mellitus	Insulin Detemir Injection	Denmark	Novo Nordisk Biotechnology Ltd	2004	2009	Class-A (2009)
	Glibenclamide Tablets	China	Zhicheng Pharmaceutical Ltd	1966	1999	Class-A (2004)
Neuromyelitis Optica	Inebilizumab Injection	Britain	AstraZeneca Pharmaceutical Ltd	2020	2022	Class-B (2022)
Niemann-Pick Disease	Miglustat Capsules	Switzerland	Actelion Pharmaceuticals Ltd	2009	2016	Class-B (2019)
Ornithine Transcarbamylase Deficiency	Sodium Phenylbutyrate	China	Zhaoke Pharmaceutical (Guangzhou) Ltd	1996	2021	-
Parkinson Disease (Young-onset , Early-onset)	Entacapone,Levodopa and Carbidopa Tablets	Finland	Orion Corporation	1998	2016	Class-B (2019)
	Rasagiline	Israel	Teva Pharmaceutical Industries Ltd	2005	2017	Class-B (2019)
	Carbidopa and levodopa Tablets	America	Merck Sharp & Dohme	1975	1991	Class-B (2004)
	Droxidopa Capsules	China	Chongqing Shenghuaxi Pharmaceutical Ltd	1989	2012	Class-B (2017)
	Amantadine Hydrochloride Tablets	China	Northeast Pharmaceutical Group Ltd	1966	1971	Class-A (2004)
	Ropinirole Hydrochloride Tablets	Britain	GlaxoSmithKline	1996	2018	Class-B (2017)
	Pramipexole Dihydrochloride Tablets	Germany	Boehringer Ingelheim Pharma GmbH & Co. KG	1999	2005	Class-B (2009)
	Selegiline Hydrochloride Tablets	Finland	Orion Corporation	1995	2004	Class-B (2004)
	Levodopa Tablets	Switzerland	Roche Pharmaceutical Ltd	1973	1997	Class-A (2004)
	Rotigotine Patches	Belgium	UCB Pharma S.A.	2006	2018	-
Paroxysmal Nocturnal Hemoglobinuria	Eculizumab Injection	Britain	AstraZeneca Pharmaceutical Ltd	2007	2018	-
Primary Combined Immune Deficiency	Human Immunolobulin (pH4) for Intravenous Injection	China	Guanfeng Biological Products Ltd	2006	2018	Class-B (2017)
Primary Light Chain Amyloidosis	Daratumumab Injection	America	Janssen-Cilag International NV.	2015	2019	Class-B (2021)
Severe Congenital Neutropenia	Mecapegfilgrastim Injection	China	Jiangsu Hengrui Pharmaceuticals Ltd	N.A.	2018	Class-B (2019)
	Lenograstim	Japan	Chugai Pharmaceutical Ltd	1991	1993	-
Sitosterolemia	Ezetimibe Tablets	America	Merck Sharp & Dohme	2002	2012	Class-B (2017)
Spinal Muscular Atrophy	Nusinersen Sodium Injection	America	Biogen, Inc.	2016	2019	Class-B (2021)
	Risdiplam Powder for Oral Solution	Switzerland	Roche Pharmaceutical Ltd	2020	2021	Class-B (2022)
Tetrahydrobiopterin Deficiency	Sapropterin Dihydrochloride Tablets	Germany	Excella GmbH & Co.KG	2007	2010	-
Tuberous Sclerosis Complex	Everolimus Tablets	Switzerland	Novartis Pharma Schweiz AG	2003	2013	Class-B (2017)
Tyrosinemia	Nitisinone	Switzerland	Swedish Orphan Biovitrum AB	2002	2021	-
X-linked Agammaglobulinemia	Human Immunolobulin (pH4) for Intravenous Injection	China	Guanfeng Biological Products Ltd	2006	2018	Class-B (2017)
Lennox-Gastaut syndrome	lamotrigine Tablets	Britain	GlaxoSmithKline	1994	1999	Class-B (2009)
Mediterranean Anemia	Desferrioxamine Mesylate for Injection	Switzerland	Novartis Pharma Schweiz AG	1975	2005	Class-A (2004)
	Deferasirox Dispersible Tablets	Switzerland	Novartis Pharma Schweiz AG	2005	2010	Class-B (2019)
Myelofibrosis	Ruxolitinib Phosphate Tablets	Switzerland	Novartis Pharma Schweiz AG	2011	2017	Class-B (2019)
Myelodysplastic syndrome	Decitabine for Injection	America	Janssen-Cilag International NV.	2006	2009	Class-B (2017)
	Azacitidine for Injection	Germany	Baxter Oncology GmbH	2004	2017	Class-B (2017)
Crohn disease	Infliximab for Injection	America	Merck Sharp & Dohme	1998	2006	Class-B (2019)
Respiratory distress syndrome	Poractant Alfa Injection	Italy	Chiesi Farmaceutici S.p.A	N.A.	2001	Class-B (2017)
Acromegaly	Bromocriptine Mesilate Tablets	Switzerland	Novartis Pharma Schweiz AG	1978	2001	Class-B (2004)

Table 1. The list of 106 marketed drugs approved in China for the treatment of rare diseases (continued)

Rare Disease	Drug	R&D Country	R&D Manufacturer	Time to market (abroad)	Time to market (China)	Medical insurance type (insurance period)
	Octreotide Acetate Microspheres for Injection	Switzerland	Novartis Pharma Schweiz AG	1998	2003	Class-B (2017)
	Lanreotide Acetate Sustained-release Injection	France	Ipsen Pharma Biotech	1995	2019	Class-B (2020)
Multiplemyeloma	Bortezomib for Injection	America	Janssen-Cilag International NV.	2003	2005	Class-B (2017)
	Lenalidomide	America	Celgene Corporation	2005	2013	Class-B (2017)
	Thalidomide	Switzerland	Novartis Pharma Schweiz AG	1957	2008	Class-B (2004)
	Ixazomib Citrate Capsules	Japan	Takeda Pharmaceutical Company Limited	2015	2018	Class-B (2017)

N.A.[*] : Not available; -[**] : none medical insurance drugs.

the same disease. For example, the greatest number of drugs (*10*) are available for treating Parkinson's disease (young-onset, early onset), of which ropinirole hydrochloride tablets and pramipexole hydrochloride tablets are dopamine agonists of the non-ergoline class, and rasagiline and selegiline hydrochloride tablets are monoamine oxidase B inhibitors. This is followed by nine drugs for treating multiple sclerosis, of which fingolimod hydrochloride capsules and siponimod tablets are both sphingosine-1-phosphate receptor modulators. There are eight drugs for treating idiopathic pulmonary hypertension, which are endothelin receptor antagonists such as ambrisentan, bosentan, and macitentan. The drugs used to treat thalassemia are deferoxamine and deferasirox dispersible tablets, and both drugs are oral iron chelators. A total of 11 orphan drugs such as droxidopa capsules, ezetimibe tablets, and hydrocortisone can be used to treat two rare diseases and that there are many drugs for treating four rare diseases, such as 21-hydroxylase deficiency and idiopathic hypogonadotropic hypogonadism (Table 3).

4. Outlook

Currently, the orphan drug landscape in China can be summarized as follows: drugs not available in China but available overseas, drugs available in China but not included in medical insurance, and drugs included in medical insurance but not available in hospitals. Although major progress has been achieved in the rare disease medical insurance system in China, it is mainly based on basic medical insurance, and charity and other security systems do not play a supplementary role. In addition, there are still a large number of orphan drugs that have not been included in the basic medical insurance directory, placing immense burdens on patients. Because of this, we formulated national rare disease security policies and directed the process of rare disease legislation. Systematic special laws should be passed for orphan drug development, review and approval, security, and services. In addition, modifications to the rare disease directory should be accelerated by refining the dynamic updating of the rare disease directory on the basis of disease prevalence, medical technology level, disease burden, and security level of China. Furthermore, we recommend the improvement of the multilayered healthcare security system for rare diseases on the basis of basic medical insurance supplemented by commercial health insurance, charity donations, and patient self-pay and the identification of the limits and responsibilities of various security entities. The government should guide social forces to set up a rare disease–related fund, lead in establishing a negotiating platform for pharmaceutical companies and commercial insurance, promote the creation of a discussion platform for inclusive commercial insurance, and include more

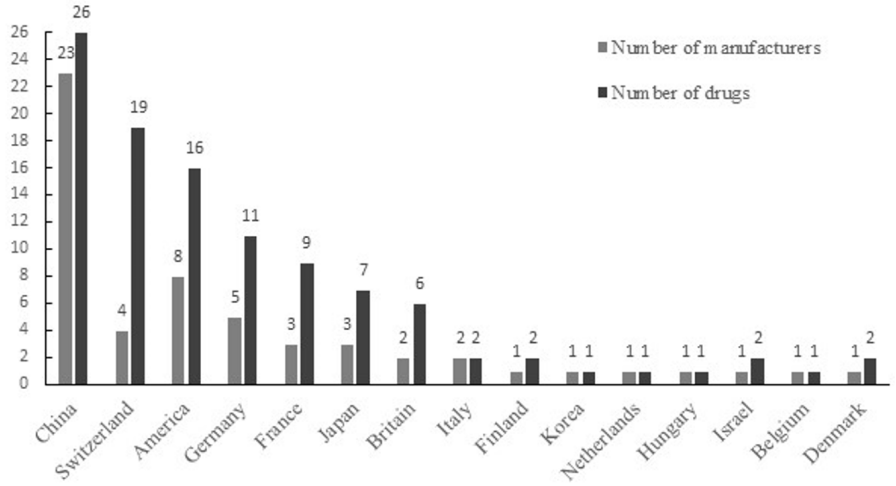


Figure 1. Distribution of R&D manufacturers for marketed 106 orphan drugs.

Table 2. The list of 106 marketed drugs approved in China for the treatment of rare diseases

Rare disease	Number of drug	Marketed drug
Parkinson Disease (Young-onset , Early-onset)	10	Entacapone,Levodopa and Carbidopa Tablets, Rasagiline, Carbidopa and levodopa Tablets, Droxidopa Capsules, Amantadine Hydrochloride Tablets, Ropinirole Hydrochloride Tablets, Pramipexole Dihydrochloride Tablets, Selegiline Hydrochloride Tablets, Levodopa Tablets, Rotigotine Patches
Multiple Sclerosis	9	Fampridine, Fingolimod Hydrochloride Capsules, Siponimod Tablets, Teriflunomide Tablets, Baclofen Tablets, Dimethyl Fumarate Enteric Capsules, Ofatumumab Injection, Interferon beta-1b, Recombinant human interferon beta-1b for injection
Hemophilia	9	Human Coagulation Factor IX, Desmopressin Acetate, Human Prothrombin Complex, Human Coagulation Factor VIII, Recombinant Human Coagulation Factor VIII, Recombinant Human Coagulation factor IX, Recombinant Human Coagulation Factor VIIa, Eftrenonacog alfa for Injection, Emicizumab Injection
Idiopathic Pulmonary Arterial Hypertension	8	Treprostinil Injection, Ambrisentan tablets, Bosentan Tablets, Riociguat Tablets, Macitentan Tablets, Selexipag Tablets, Sildenafil Citrate Tablets, Iloprost
Hepatolenticular Degeneration	4	Penicillamine Tablets, Sodium Dimercaptosuccinate, Dimercaptosuccinic Acid, Zinc Sulfate
Multiplemyeloma	4	Bortezomib for Injection, Lenalidomide, Thalidomide, Ixazomib Citrate Capsules
Hereditary Angioedema	3	Danazol Capsules, Icatibant Acetate Injection, Lanadelumab Injection
Idiopathic Hypogonadotropic Hypogonadism	3	Gonadorelin for Injection, Menotrophins for Injection, Chorionic Gonadotrophin for Injection
Kallmann Syndrome	3	Gonadorelin for Injection, Menotrophins for Injection, Chorionic Gonadotrophin for Injection
Amyotrophic Lateral Sclerosis	3	Riluzole Oral Suspension, Edaravone Injection, Riluzole Tablets
Homozygous Hypercholesterolemia	3	Evolocumab Injection, Rosuvastatin Calcium Tablets, Ezetimibe Tablets
Acromegaly	3	Bromocriptine Mesilate Tablets, Octreotide Acetate Microspheres for Injection, Lanreotide Acetate Sustained-release Injection (a pre-filled syringe)
Mucopolysaccharidosis	3	Laronidase concentrated solution for infusion, Elosulfase alfa injection, Idursulfase beta Injection
Neonatal Diabetes Mellitus	2	Insulin Detemir Injection, Glibenclamide Tablets
Idiopathic Pulmonary Fibrosis	2	Nintedanib esilate soft capsules, Pirfenidone Capsules
Mediterranean Anemia	2	Desferrioxamine Mesylate for Injection, Deferasirox Dispersible Tablets
Myelodysplastic syndrome	2	Decitabine for Injection, Azacitidine for Injection
Spinal Muscular Atrophy	2	Nusinersen Sodium Injection, Risdiplam Powder for Oral Solution
Severe Congenital Neutropenia	2	Mecapegfilgrastim Injection, Lenograstim
Huntington Disease	2	Deutetrabenazine Tablets, Tetrabenazine
Hereditary Hypomagnesemia	2	Magnesium Gluconate, Potassium Aspartate and Magnesium Aspartate
Gaucher's Disease	2	Velaglucerase Alfa for Injection, Imiglucerase for Injection
Fabry Disease	2	Agalsidase Alfa Concentrated Solution for Infusion, Agalsidase Beta for Injection
21-Hydroxylase Deficiency	2	Hydrocortisone, Hydrocortisone Acetate
Congenital Adrenal Hypoplasia	2	Hydrocortisone, Hydrocortisone Acetate

Table 3. Marketed drugs for treatment of multiple rare diseases

Marketed drug	Rare disease
Droxidopa Capsules	Parkinson Disease (Young-onset , Early-onset), Multiple System Atrophy
Ezetimibe Tablets	Homozygous Hypercholesterolemia, Sitosterolemia
Sapropterin Dihydrochloride Tablets	Hyperphenylalaninemia, Tetrahydrobiopterin Deficiency
Human Immunolobulin (pH4) for Intravenous Injection	X-linked Agammaglobulinemia, Primary Combined Immune Deficiency
Eculizumab Injection	Atypical Hemolytic Uremic Syndrome, Paroxysmal Nocturnal Hemoglobinuria
Levocarnitine	Carnitine Deficiency, Beta-ketothiolase Deficiency
Hydrocortisone, Hydrocortisone Acetate	22-Hydroxylase Deficiency, Congenital Adrenal Hypoplasia
Gonadorelin for Injection, Menotrophins for Injection, Chorionic Gonadotrophin for Injection	Idiopathic Hypogonadotropic Hypogonadism, Kallmann Syndrome

orphan drugs in the commercial insurance directory. At the same time, secondary reimbursement can be carried out through major disease insurance or medical insurance reimbursement to further decrease the medication burden on patients.

Funding: This work was supported by a grant from Natural Science Foundation of Shandong Province (General program ZR2023MH276) and Academic Promotion Program of Shandong First Medical University (LJ001).

Conflict of Interest: The authors have no conflicts of interest to disclose.

References

1. Lu J, Pan X. Current situation and future of rare diseases in China. China Tropical Medicine. 2023; 23:109-114. (in Chinese)
2. Chung CCY; Hong Kong Genome Project; Chu ATW, Chung BHY. Rare disease emerging as a global public health priority. Front Public Health. 2022; 10:1028545.
3. Gopal-Srivastava R, Kaufmann P. Facilitating clinical studies in rare diseases. Adv Exp Med Biol. 2017; 1031:125-140.
4. FDA Newsroom. Taking New Steps to Meet the Challenges of Rare Diseases — FDA Marks the 11th Rare Disease Day. *https://www.fda.gov/news-events/fda-voices/taking-new-steps-meet-challenges-rare-diseases-fda-marks-11th-rare-disease-day* (accessed March 14, 2023).
5. European Medicines Agency. Market exclusivity: orphan medicines. *https://www.ema.europa.eu/en/human-regulatory/post-authorisation/orphan-medicines/market-exclusivity-orphan-medicines* (accessed March 14, 2023).
6. National Medical Products Administration, China. Implementation Regulations of the Drug Administration Law of the People's Republic of China (Draft Revisions). *https://www.nmpa.gov.cn/xxgk/zhqyj/zhqyjyp/20220509220456183.html* (accessed March14, 2023). (in Chinese)
7. General Office of the State Council. Notice of the General Office of the State Council on Printing and Distributing the "14th Five-Year Plan" National Health Plan. *http://www.gov.cn/zhengce/content/2022-05/20/content_5691424.htm* (accessed March 14, 2023). (in Chinese)
8. National Health Commission, China. Notice of the General Office of the National Health Commission on Carrying out the Registration of Diagnosis and Treatment Information for Rare Disease Cases. *http://www.gov.cn/zhengce/zhengceku/2019-11/13/content_5451656.htm* (accessed March 14, 2023). (in Chinese)
9. Zhejiang Provincial Medical Insurance Bureau. Notice on Establishing a Drug Guarantee Mechanism for Rare Diseases in Zhejiang Province. *http://ybj.zj.gov.cn/art/2019/12/31/art_1229113757_601196.html* (accessed March 14, 2023). (in Chinese)
10. Chengdu Medical Security Bureau, Chengdu Municipal Finance Bureau, Chengdu Municipal Health Commission. "Notice on Issues Related to Drug Support for Rare Diseases" issued by Chengdu Medical Security Bureau, Chengdu Municipal Finance Bureau and Chengdu Municipal Health Commission. *http://cdyb.chengdu.gov.cn/ylbzj/c128998/2021-03/18/content_ebb0abea709e46999e3bddd5b24834d1.shtml* (accessed March14, 2023). (in Chinese)
11. The People's Government of Foshan Municipality. Notice of the Office of Foshan Municipal People's Government on Printing and Distributing the Medical Assistance Measures of Foshan City. *http://www.foshan.gov.cn/zwgk/rdzt/lwlb/zcjd/content/post_4516293.html* (accessd March 14, 2023). (in Chinese)
12. National Health Commission, Ministry of Science and Technology, Ministry of Industry and Information Technology, National Medical Products Administration, National Administration of Traditional Chinese Medicine. Notice on Publishing the First Batch of Rare Diseases Catalogue. *http://www.gov.cn/zhengce/zhengceku/2018-12/31/content_5435167.htm* (accessed March 14, 2023). (in Chinese)
13. Chinese Communist Party Central Committee, General Office of the State Council. Opinions of the Chinese Communist Party Central Committee and General Office of the State Council on Deepening the Reform of the Medical Security System. *http://www.gov.cn/zhengce/2020-03/05/content_5487407.htm* (accessed March 14, 2023). (in Chinese)
14. National Healthcare Security Administration. The National Healthcare Security Administration announcedon the "Work Plan for the Adjustment of the National Basic Medical Insurance, Industrial Injury Insurance, and Maternity Insurance Drug Catalog in 2022" and announcement of relevant documents. *http://www.gov.cn/zhengce/zhengceku/2022-06/30/content_5698559.htm* (accessed March 14, 2023). (in Chinese)
15. National Healthcare Security Administration, Ministry

of Human Resources and Social Security. Notice of the National Healthcare Security Administration and the Ministry of Human Resources and Social Security on Printing and Distributing the "Catalogue of National Basic Medical Insurance, Industrial Injury Insurance, and Maternity Insurance Drugs (2022)". *http://www.nhsa.gov.cn/art/2023/1/18/art_104_10078.html* (accessed March 14, 2023). (in Chinese)

Received April 13, 2023; Revised July 23, 2023; Accepted August 6, 2023.

**Address correspondence to:*
Yanqin Lu and Jinxiang Han, Shandong First Medical University & Shandong Academy of Medical Sciences, No. 6699 Qingdao Road, Ji'nan, 250117, China.
E-mail: yqlu@sdfmu.edu.cn (YL), jxhan@sdfmu.edu.cn (JH)

Released online in J-STAGE as advance publication August 12, 2023.

Review

DOI: 10.5582/irdr.2023.01047

Ortner's syndrome: A systematic review of presentation, diagnosis and management

Sameer Verma[1], Ankoor Talwar[2], Abhinav Talwar[3], Sarah Khan[1], Kambhampaty Venkata Krishnasastry[4,5], Arunabh Talwar[1,5,*]

[1] Division of Pulmonary Medicine, Northshore University Hospital, Manhasset, NY, USA;
[2] Albany Medical College, Albany, NY, USA;
[3] Feinberg School of Medicine, Chicago, IL, USA;
[4] Department of Surgery, Northwell Health, New York, NY, USA;
[5] Donald and Barbara Zucker School of Medicine, Hempstead, NY, USA.

SUMMARY Ortner's syndrome (OS), also called cardiovocal syndrome, is a rare condition hallmarked by left recurrent laryngeal nerve palsy due to underlying cardiopulmonary disease. The purpose of this review is to systemically analyze the existing literature for cases of OS to outline typical presentation, methods of diagnosis, and management of these patients. Case reports, case series, and cohort studies describing OS between 1955 and 2021 were identified. Individual manuscripts were reviewed for clinical features, presentation, and management. A total of 117 patient cases were gathered from 92 published articles. Common symptoms included hoarseness, dyspnea, cough, and dysphagia. The most common associated comorbidity was aortic aneurysm (41%), followed by pulmonary hypertension (35%), mitral stenosis (17%), and hypertension (12%). Among those who were managed *via* surgical intervention, 85.4% reported improvement in their hoarseness. While historically OS was associated with mitral stenosis, in recent decades, aortic aneurysms and dilation of the pulmonary artery from pulmonary hypertension have emerged as primary etiologies of OS. Therefore, OS should be considered in any patient presenting with hoarseness and history of cardiopulmonary disease. Surgical intervention in appropriate candidates resolves OS in most cases.

Keywords Ortner's syndrome, cardiovocal syndrome, hoarseness

1. Introduction

Vocal cord paralysis presenting as hoarseness due to an underlying cardiovascular pathology is a rare clinical entity known as Ortner's syndrome (OS) or cardiovocal syndrome. The syndrome was first described by Norbert Ortner in 1897 in a review of three patients with severe mitral stenosis (*1*). It was postulated that left atrial enlargement in these patients was responsible for left vocal cord paralysis and subsequent dysphonia.

The pathophysiology of OS is related to the anatomy of the recurrent laryngeal nerve (RLN). The RLN is a branch of the vagus nerve (cranial nerve X) that innervates all the intrinsic muscles of larynx except for the cricothyroid muscle. These muscles act to open, close, and adjust tension on the vocal cords bilaterally. The RLN is also responsible for the sensory supply to the larynx below the vocal cords and the upper part of the trachea. The pathways for the RLN on both sides of the neck are asymmetrical. The right recurrent laryngeal nerve branches off the right vagus nerve, loops around right subclavian artery and tracks superiorly between the trachea and esophagus. On the other hand, the left recurrent laryngeal nerve branches off the left vagus nerve, loops around the ligamentum arteriosum and tracks superiorly between the trachea and the esophagus (Figure 1). In general, injury to left recurrent laryngeal nerve (*i.e.* impingement, stretching, or compression) is more common than injury to the right recurrent laryngeal nerve, likely due to its proximity to the aortopulmonary window and other intrathoracic structures (*2*).

OS is specific for left recurrent laryngeal nerve injury due to underlying cardiac disease. Although it is commonly associated with severe mitral stenosis (as initially described by Ortner), there are many causes of OS including compression from other vascular (*i.e.* aortic aneurysms, aortic dissections, pulmonary hypertension) or mediastinal (*i.e.* neoplasms) structures (*3-5*). Similarly, although the classic symptom associated with OS is dysphonia/hoarseness, there have been several other

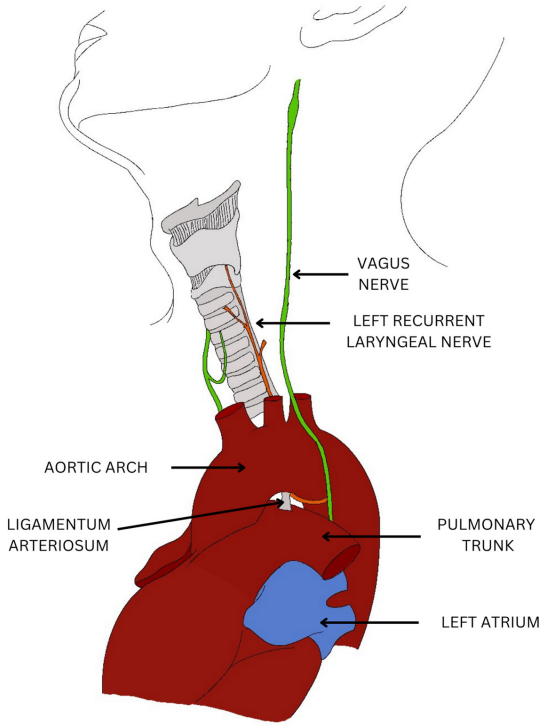


Figure 1. Anatomic pathway of the left recurrent laryngeal nerve.

manifestations of the syndrome described in the literature including aspiration, dysphagia, and shortness of breath (*6*).

As OS is a rare clinical entity, there is a paucity of literature comprehensively describing the spectrum of clinical manifestations as well as etiologies of the syndrome. As such, the purpose of this manuscript is to systemically review the existing literature for cases of OS to outline the syndrome's various etiologies, symptomatology, methods of diagnosis, and management strategies.

2. Systematic review

This systematic review was conducted in line with the PRISMA 2020 guidelines. As this is a review of existing literature, institutional ethics approval was not required by our institutional review board. A PubMed search was performed by two authors (SV, SK) to find articles published between 1955 and 2021 using the keywords "Ortner's syndrome" or "cardiovocal syndrome". Case reports, case series, and cohort studies were included. The references lists of articles were also reviewed to find additional relevant literature. Only English language literature was included in the analysis. Manuscripts were excluded if they described right recurrent laryngeal nerve palsy or if they described idiopathic left recurrent laryngeal nerve palsy. Full text review was subsequently conducted of all remaining studies for completeness of information. Any disagreements were resolved by a third reviewer (AT). Literature that met inclusion criteria was reviewed for patient clinicodemographic data, disease presentation, diagnosis, and management.

3. Main findings

Figure 2 is a PRISMA diagram depicting the literature search process. After omitting duplicate literature, a total of 188 records were included in our initial search. Of these publications, only 92 ultimately fulfilled inclusion criteria (Supplemental Table 1, *http://www.irdrjournal.com/action/getSupplementalData.php?ID=166*), encompassing a total of 117 patient cases (*1,3,6-95*). Most patients were older than 50 years of age (n = 67, 57%) (Table 1). The mean age was 53.3 years ± 34.6 years, with a range of 1.4 years to 89 years of age. Of note, the age of one patient was not presented and two patients were described as toddlers (without a specific age). There were 66 males (56%) and 50 females (44%), with one patient of unknown sex.

3.1. Clinical presentation

The most common clinical presentation in OS was hoarseness of voice, which was found in 101 patients (86.3%). Hoarseness varied in severity, with the onset described as either gradual or sudden. Other common symptoms on initial presentation were dyspnea (n = 47, 40.1%), cough (n = 15, 12.8%), and dysphagia (n = 15, 28.8%). Less common presenting symptoms are further described in Table 2. On examination, 40 patients (34.2%) had audible murmurs. The most common comorbidity described was aortic aneurysm (n = 48, 41%), followed by pulmonary hypertension (n = 41, 35%), mitral stenosis (n = 20, 17%), and hypertension (n = 14, 12%). Less common comorbidities are tabulated in Table 3.

3.2. Diagnostic workup

There were several modalities reported in the workup of OS. The most common modality was laryngoscopy to visualize vocal cord dysfunction (n = 90, 77%). In other patients, cardiovascular and mediastinal abnormalities were noted on CT scan (n = 72, 62%), chest x-ray (n = 63, 59%), and echocardiography (n = 39, 33%). Among all patients, the most common mediastinal abnormalities were thoracic aortic aneurysm (n = 48, 41%), pulmonary artery dilatation (n = 36, 31%), cardiomegaly (n = 31, 26%) and left atrial enlargement (n = 30, 27%) (Table 4). Interestingly, 38 patients (32.4%) had abnormal EKGs on initial evaluation.

3.3. Management

In total, 41 patients (35.0%) received some form of surgical intervention. Another 44 patients (37.6%) received conservative treatment, including non-surgical therapies. It was unknown whether the final 32 patients

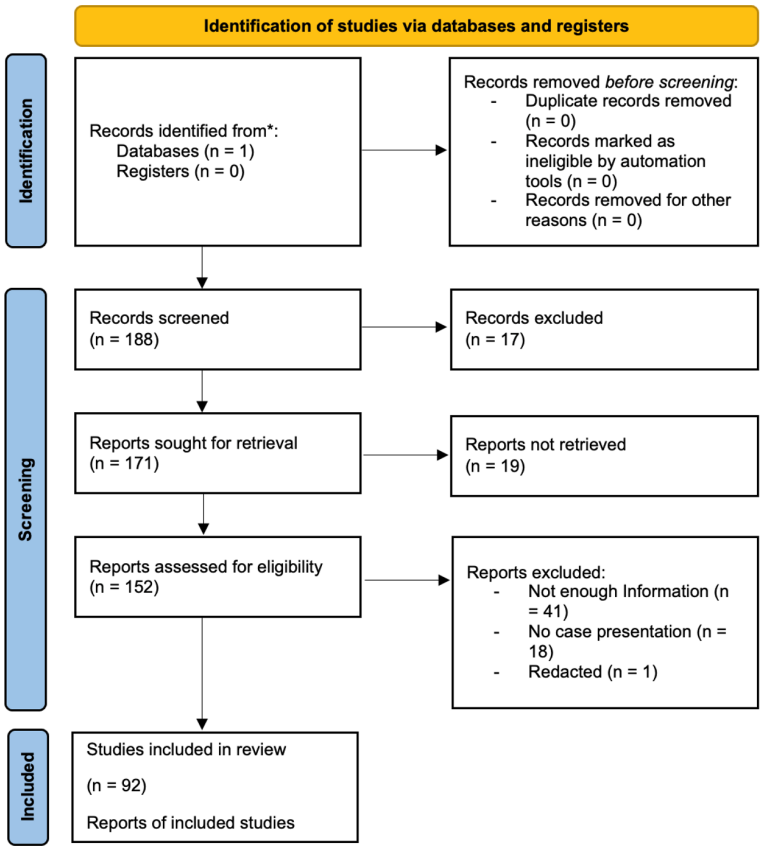


Figure 2. PRISMA flow diagram for article selection.

Table 1. Demographic characteristics of included patients

Characteristic	Number of patients	Percentage of patients
Sex[a]		
Male	66	56.90%
Female	50	43.10%
Age (years)[b]		
<10	2	1.75%
11-19	7	6.14%
20-29	13	11.40%
30-39	15	13.16%
40-49	10	8.77%
50-59	13	11.40%
60-69	18	15.79%
70-79	21	18.42%
80-89	15	13.16%

[a]: the sex of one patient was not reported; [b]: the ages of three patients were not reported.

Table 2. Clinical findings for patients with Ortner's syndrome

Clinical symptom	Number of patients	Percentage of patients
Hoarseness of voice	101	86.32%
Dyspnea	46	39.32%
Murmur	40	34.19%
Cough	15	12.82%
Dysphagia	14	11.97%
Edema	11	9.40%
Dysphonia	10	8.55%
Chest Pain	6	5.13%
Hemoptysis	6	5.13%

Table 3. Past medical history of included patients

Comorbidities	Number of patients	Percentage of patients
Pulmonary hypertension	41	35.04%
Mitral stenosis	20	17.09%
Hypertension	14	11.97%
Congenital heart disease	13	11.11%
Connective tissue disease	5	4.27%
COPD	5	4.27%

Table 4. Radiologic findings of included patients

Finding	Number of patients	Percentage of patients
Aortic aneurysm	48	41.03%
Cardiomegaly	31	26.50%
PA dilation	36	30.77%
LA enlargement	30	25.64%

(27.4%) received surgical treatment or not. The type of surgical intervention was wide-ranging and considered based on the underlying comorbidity. In cases where an aneurysm was determined to be a cause of OS, open aortic repair or thoracic endovascular aortic repair (TEVAR) were the most common treatment options considered. In patients with congenital heart disease, such as atrial septal defect (ASD) or ventricular septal defect (VSD), surgical closure of these aberrations was performed. Some other procedures were reported as well such as medialization of vocal cords, thyroplasty and angioplasty. Ultimately, 35 patients out of the 41 who were operated on reported improvement in their hoarseness after surgical treatment (85.4%).

4. Discussion and evaluation

Left recurrent laryngeal nerve injury can lead to unilateral vocal cord paralysis and hoarseness of voice. The differential diagnosis of this condition is far and wide. When this occurs due to underlying cardiovascular pathology, it is known as Ortner's syndrome or cardiovocal syndrome. Though first described in 1897 by Nobert Ortner, only a handful of cases have been reported on in the literature over the last half-century. The present analysis is a conglomeration of the reported cases in the literature and elucidates several key characteristics surrounding the diagnosis and management of Ortner's syndrome.

Most saliently, this review shows that OS can occur in patients of all ages and in patients with many different cardiovascular pathologies. Early on, mitral stenosis was thought to be the primary cause of OS (*7*). In fact, our analysis found that the most common cause of OS in the literature between 1955 and 1990 was mitral stenosis. However, from the 1990s onwards, vascular lesions (particularly thoracic aortic aneurysms) were the most common cause of OS among the cases included in this analysis. This epidemiological shift may be due to improved early detection and treatment of mitral stenosis.

Taking all patients into consideration, we found that the most common etiology of OS in the literature is thoracic aortic aneurysm (41%) as opposed to left atrial enlargement (26%). This is in line with Yuan SM's work which reported that left atrial enlargement accounts for approximately 8% of all reported cases of OS (*96*). In general aortic aneurysm related to any etiology (traumatic (*97*), mycotic (*98*), dissecting aneurysm (*99*), infection (*100*) have all been related with this condition. Thus, it is important for senior surgeons to suspect aortic aneurysm in cases of de novo hoarseness.

Interestingly, cardiovascular pathologies affecting the great vessels aside from the left heart and aorta can also lead to OS. For example, the present review includes several cases of OS in patients with pulmonary artery dilation, in the setting of either primary pulmonary hypertension (*54*) or secondary pulmonary hypertension associated with chronic thromboembolism (*58,101*). In these patients, OS manifests when the dilated pulmonary artery compresses the recurrent laryngeal nerve against the aorta. We also found 13 cases of OS that were associated with congenital heart disease. These conditions included ASD, VSD, Ebstein's anomaly, patent ductus arteriosus with or without associated aneurysm (*25*), and double outlet right ventricle associated with aortopulmonary window. It is possible that these patients developed Eisenmenger syndrome resulting in compression of the left recurrent laryngeal nerve against the aorta.

Given the rarity of OS, it is generally not part of the initial differential diagnosis of hoarseness of voice. Therefore, to confirm a diagnosis of OS, one must have a high index of suspicion, and, at times, multiple diagnostic imaging modalities are required. In patients presenting with hoarseness, a chest x-ray is usually the primary imaging study ordered which can emphasize any underlying condition such as a lung mass or cardiomegaly. Besides an x-ray, symptoms of hoarseness should also propagate a referral to specialist for laryngoscopy, which most of the patients in our analysis received. Laryngoscopy confirms the presence of vocal cord dysfunction although VC palsy can also be seen on the CT of the neck (*102*). Echocardiography is one of the most routine procedures done in to evaluate structural integrity of cardiovascular system, and is an important consideration given that cardiovascular abnormalities seem to be the most common etiology of OS. Regardless, a prompt diagnosis is critical because the underlying condition can be a risk factor for other complications such as dysphagia and/or airway obstruction.

Treatment of OS entails direct management of the underlying condition. Since hoarseness is the main presenting symptom, the prospect of recovery from hoarseness is dependent on its duration and severity (*45*). The decision to intervene is also dependent on other factors such co-morbidities, surgical risk, and patient's readiness. Indeed, 27% of our patients opted for non-surgical management, indicating how important it is for providers to assess each patient's case. In this group, patients were managed with conservative management or no treatment at all. Still, among the patients who pursued treatment, 35 (85%) had improvement in symptomatology, indicating that OS is a potentially reversible syndrome.

In conclusion, OS is a rare condition and can remain undiagnosed for a long period of time. Hoarseness is its landmark presenting symptom. It can present in any age group, but suspicion should be high if a patient has history of pulmonary hypertension or cardiac pathology. From a historical perspective, mitral stenosis was considered a primary cause of the syndrome. However, in the last two decades, aortic arch aneurysms and dilation of the pulmonary artery from pulmonary hypertension

have emerged as a primary etiology. In either case, heart murmur is the most common physical exam sign and the decision to intervene depends on many factors, such as age, comorbidity, and patient willingness. Notably, surgical intervention resolves OS in most cases.

Funding: None.

Conflict of Interest: The authors have no conflicts of interest to disclose.

References

1. Dutra BL, Campos Lda C, Marques Hde C, Vilela VM, Carvalho RE, Duque AG. Ortner's syndrome: Acase report and literature review. Radiol Bras. 2015; 48:260-262.
2. Loughran S, Alves C, MacGregor FB. Current aetiology of unilateral vocal fold paralysis in a teaching hospital in the West of Scotland. J Laryngol Otol. 2002; 116:907-910.
3. Coen M, Leuchter I, Sussetto M, Banfi C, Giraud R, Bendjelid K. Progressive dysphonia: Ortner syndrome. Am J Med. 2018; 131:e494-e495.
4. Ismazizi Z, Zainal AA. Thoracic aortic aneurysm as a cause of Ortner's syndrome - A Case series. Med J Malaysia. 2016; 71:139-141.
5. Shankar O, Lohiya BV. Cardiovocal syndrome--a rare presentation of primary pulmonary hypertension. Indian Heart J. 2014; 66:375-377.
6. Arifputera A, Loo G, Chang P, Kojodjojo P. An unusual case of dysphonia and dysphagia. Singapore Med J. 2014; 55:e31-33.
7. Fife R, Muir A. Laryngeal paralysis associated with mitral stenosis (Ortner's syndrome): report of two cases. Glasgow Med J. 1955; 36:164-167.
8. Camishion RC, Gibbon JH, Jr., Pierucci L, Jr. Paralysis of the left recurrent laryngeal nerve secondary to mitral valvular disease. Report of two cases and literature review. Ann Surg. 1966; 163:818-828.
9. Pai KN, Kini PM, Chandrasekhar KP. Ortner's syndrome. A report of 2 cases. Indian Heart J. 1966; 18:407-410.
10. Vigg BL, Sahay BK, Dandilliya R. Ortner's syndrome. J Assoc Physicians India. 1970; 18:379-381.
11. D'Agostino N, De Ritis G. On an unusual case of so-called opaque hemithorax with acute bronchopneumonic pathogenesis. Lotta Tuberc. 1971; 41:109-112 passim. (in Italian)
12. Solanki SV, Yajnik VH. Ortner's syndrome. Indian Heart J. 1972; 24:43-46.
13. Agrawal JK, Agrawal BV. Silent mitral stenosis with Ortner's syndrome. J Assoc Physicians India. 1973; 21:387-390.
14. Sharma NG, Kapoor CP, Mahambre L, Borkar MP. Ortner's syndrome. J Indian Med Assoc. 1973; 60:427-429 passim.
15. Kagal AE, Shenoy PN, Nair KG. Ortner's syndrome associated with primary pulmonary hypertension. J Postgrad Med. 1975; 21:91-95.
16. Karhade NV, Bhagwat RB. Mitral valve disease associated with tracheal tug and Ortner's syndrome. J Med Soc N J. 1975; 72:937-939.
17. Victor S, Daniel ID, Santosham R, Rajaram S, Hussain AT, Ramadoss T. Ortner's syndrome following intracardiac repair for tetralogy of Fallot. Indian Heart J. 1978; 30:306-308.
18. Bahl DV, Vaidya MP, Khanna MN. Ortner's syndrome. Case report. Indian Heart J. 1979; 31:176-181.
19. Nakao M, Sawayama T, Samukawa M, Mitake H, Nezuo S, Fuseno H, Hasegawa K. Left recurrent laryngeal nerve palsy associated with primary pulmonary hypertension and patent ductus arteriosus. J Am Coll Cardiol. 1985; 5:788-792.
20. Chan P, Lee CP, Ko JT, Hung JS. Cardiovocal (Ortner's) syndrome left recurrent laryngeal nerve palsy associated with cardiovascular disease. Eur J Med. 1992; 1:492-495.
21. Thirlwall AS. Ortner's syndrome: a centenary review of unilateral recurrent laryngeal nerve palsy secondary to cardiothoracic disease. J Laryngol Otol. 1997; 111:869-871.
22. Sengupta A, Dubey SP, Chaudhuri D, Sinha AK, Chakravarti P. Ortner's syndrome revisited. J Laryngol Otol. 1998; 112:377-379.
23. Khan IA, Wattanasauwan N, Ansari AW. Painless aortic dissection presenting as hoarseness of voice: cardiovocal syndrome: Ortner's syndrome. Am J Emerg Med. 1999; 17:361-363.
24. Kishan CV, Wongpraparut N, Adeleke K, Frechie P, Kotler MN. Ortner's syndrome in association with mitral valve prolapse. Clin Cardiol. 2000; 23:295-297.
25. Day JR, Walesby RK. A spontaneous ductal aneurysm presenting with left recurrent laryngeal nerve palsy. Ann Thorac Surg. 2001; 72:608-609.
26. Foster PK, Astor FC. Vocal fold paralysis in painless aortic dissection (Ortner's syndrome). Ear Nose Throat J. 2001; 80:784.
27. Hebl JR, Rose SH, Narr BJ, Rorie DK. Postoperative left vocal cord dysfunction caused by Ortner's cardiovocal syndrome. Anesth Analg. 2001; 92:1071-1072.
28. Bickle IC, Kelly BE, Brooker DS. Ortner's syndrome: a radiological diagnosis. Ulster Med J. 2002; 71:55-56.
29. Annema JT, Brahim JJ, Rabe KF. A rare cause of Ortner's syndrome (cardiovocal hoarseness). Thorax. 2004; 59:636.
30. Mohamed AL, Zain MM. Hoarseness of Voice in a Patient with Mitral Stenosis and Ortner's Syndrome. Malays J Med Sci. 2004; 11:65-68.
31. Panwar SS, Mehta AK, Verma RK, Mukherji B. High Altitude induced Ortner's Syndrome. Med J Armed Forces India. 2004; 60:182-183.
32. Stoob K, Alkadhi H, Lachat M, Wildermuth S, Pfammatter T. Resolution of hoarseness after endovascular repair of thoracic aortic aneurysm: a case of Ortner's syndrome. Ann Otol Rhinol Laryngol. 2004; 113:43-45.
33. Phua GC, Eng PC, Lim SL, Chua YL. Beyond Ortner's syndrome--unusual pulmonary complications of the giant left atrium. Ann Acad Med Singap. 2005; 34:642-645.
34. Wiebe S, Yoo SJ, Shroff M. Answer to case of the month #102. Ortner's syndrome (cardiovocal syndrome). Can Assoc Radiol J. 2005; 56:173-174.
35. Escribano JF, Carnes J, Crespo MA, Anton RF. Ortner's syndrome and endoluminal treatment of a thoracic aortic aneurysm: a case report. Vasc Endovascular Surg. 2006; 40:75-78.
36. Lee SI, Pyun SB, Jang DH. Dysphagia and hoarseness associated with painless aortic dissection: A rare case of cardiovocal syndrome. Dysphagia. 2006; 21:129-132.
37. Lydakis C, Thalassinos E, Apostolakis S, Athousakis E, Michou E, Kontopoulou E. Hoarseness as imminent symptom of aortic aneurysm rupture (Ortner's syndrome). Int Angiol. 2006; 25:231-233.

38. Mathai J, Swapna UP. Hoarseness -As a presenting feature of aortic arch aneurysm. Indian J Otolaryngol Head Neck Surg. 2006; 58:309-310.
39. Gulel O, Elmali M, Demir S, Tascanov B. Ortner's syndrome associated with aortic arch aneurysm. Clin Res Cardiol. 2007; 96:49-50.
40. Wunderlich C, Wunderlich O, Tausche AK, Fuhrmann J, Boscheri A, Strasser RH. Ortner's syndrome or cardiovocal hoarseness. Intern Med J. 2007; 37:418-419.
41. Fennessy BG, Sheahan P, McShane D. Cardiovascular hoarseness: An unusual presentation to otolaryngologists. J Laryngol Otol. 2008; 122:327-328.
42. Gothi R, Ghonge NP. Case Report: Spontaneous aneurysm of ductus arteriosus: A rare cause of hoarseness of voice in adults. Indian J Radiol Imaging. 2008; 18:322-323.
43. Kokotsakis J, Misthos P, Athanassiou T, Skouteli E, Rontogianni D, Lioulias A. Acute Ortner's syndrome arising from ductus arteriosus aneurysm. Tex Heart Inst J. 2008; 35:216-217.
44. Vlachou PA, Karkos CD, Vaidhyanath R, Entwisle J. Ortner's syndrome: An unusual cause of hoarse voice. Respiration. 2008; 75:459-460.
45. Chen RF, Lin CT, Lu CH. Ortner's syndrome--a rare cause of unilateral vocal cord paralysis: a case report. Kaohsiung J Med Sci. 2009; 25:203-206.
46. Yuan SM, Jing H. Cardiovocal syndrome secondary to an aortic pseudoaneurysm. Vasa. 2009; 38:382-389.
47. Lambertucci JR, Prata PH, Voieta I. Left recurrent laryngeal palsy (Ortner's syndrome) in schistosomal pulmonary hypertension. Rev Soc Bras Med Trop. 2010; 43:608.
48. Mickus TJ, Mueller J, Williams R. An uncommon cause of Ortner syndrome. J Thorac Imaging. 2010; 25:W82-84.
49. Plastiras SC, Pamboucas C, Zafiriou T, Lazaris N, Toumanidis S. Ortner's syndrome: a multifactorial cardiovocal syndrome. Clin Cardiol. 2010; 33:E99-100.
50. Van Melle JP, Meyns B, Budts W. Ortner's syndrome, presentation of two cases with cardiovocal hoarseness. Acta Cardiol. 2010; 65:703-705.
51. Garrido JM, Esteban M, Lara J, Rodriguez-Vazquez JF, Verdugo-Lopez S, Lopez-Checa S. Giant aortic arch aneurysm and cardio-vocal syndrome: Still an open-surgery Indication. Cardiol Res. 2011; 2:304-306.
52. Lin WS, Cheng CA. Ortner's Syndrome. Acta Neurol Taiwan. 2011; 20:281-282.
53. Prada-Delgado O, Barge-Caballero E. Images in clinical medicine. Ortner's syndrome. N Engl J Med. 2011; 365:939.
54. Subramaniam V, Herle A, Mohammed N, Thahir M. Ortner's syndrome: Case series and literature review. Braz J Otorhinolaryngol. 2011; 77:559-562.
55. Eccles SR, Banks J, Kumar P. Ascending aortic aneurysm causing hoarse voice: A variant of Ortner's syndrome. BMJ Case Rep. 2012; 2012.
56. Edrees A. Ortner's syndrome as a presenting feature of giant cell arteritis. Rheumatol Int. 2012; 32:4035-4036.
57. Gupta P, Sharma S. Ortner's syndrome secondary to aortic aneurysm. Ann Acad Med Singap. 2012; 41:40-41.
58. Heikkinen J, Milger K, Alejandre-Lafont E, Woitzik C, Litzlbauer D, Vogt JF, Klussmann JP, Ghofrani A, Krombach GA, Tiede H. Cardiovocal syndrome (Ortner's syndrome) associated with chronic thromboembolic pulmonary hypertension and giant pulmonary artery aneurysm: Case report and review of the literature. Case Rep Med. 2012; 2012:230736.
59. Matteucci ML, Rescigno G, Capestro F, Torracca L. Aortic arch patch aortoplasty for Ortner's syndrome in the age of endovascular stented grafts. Tex Heart Inst J. 2012; 39:401-404.
60. Sawa A, Shirokawa T, Kobayashi H, Satoh H. Ortner's syndrome in a patient with COPD. Intern Med. 2012; 51:2059.
61. Andjelkovic K, Kalimanovska-Ostric D, Djukic M, Vukcevic V, Menkovic N, Mehmedbegovic Z, Topalovic M, Tesic M. Two rare conditions in an Eisenmenger patient: Left main coronary artery compression and Ortner's syndrome due to pulmonary artery dilatation. Heart Lung. 2013; 42:382-386.
62. Matsuo S, Matsuda E, Suzuki T. Ortner's syndrome associated with hypertensive cardiac disease. Auris Nasus Larynx. 2013; 40:420-421.
63. Okoye O, Anusim N, Shon A, Mirrakhimov AE. Ortner's syndrome: a rare cause of hoarseness. BMJ Case Rep. 2013; 2013.
64. Ozyurtlu F, Acet H, Bilik MZ, Tasal A. [Ortner's syndrome caused by dissecting aortic aneurysm]. Turk Kardiyol Dern Ars. 2013; 41:225-227. (in Turkish)
65. Ali Shah MU, Siddiqi R, Chaudhri MS, Khan AA, Chaudhry I. Aortic aneurysm: a rare cause of Ortner's syndrome. J Coll Physicians Surg Pak. 2014; 24:282-284.
66. Hurtarte Sandoval AR, Carlos Zamora R, Gomez Carrasco JM, Jurado Ramos A. Ortner's syndrome: A case report and review of the literature. BMJ Case Rep. 2014; 2014.
67. Shahul HA, Manu MK, Mohapatra AK, Magazine R. Ortner's syndrome. BMJ Case Rep. 2014; 2014.
68. Gnagi SH, Howard BE, Hoxworth JM, Lott DG. Acute contained ruptured aortic aneurysm presenting as left vocal fold immobility. Case Rep Otolaryngol. 2015; 2015:219090.
69. Gorospe L, Fernandez-Mendez MA, Ayala-Carbonero AM, Garcia-Poza J, Gonzalez-Gordaliza C. Ortner's syndrome secondary to a huge left atrium. Ann Thorac Surg. 2015; 100:732.
70. Handa A, Chadha DS, Singh S, Singh N. Ortner's syndrome: A rare cause in an elderly smoker. Med J Armed Forces India. 2015; 71:S178-180.
71. Maskey-Warzechowska M, Dabrowska M, Krenke R, Domeracka-Kolodziej A, Zukowska M, Chazan R. Left brachiocephalic vein stenosis and infectious aortitis: two unusual causes of Ortner's syndrome. Pneumonol Alergol Pol. 2015; 83:457-461.
72. Pathirana U, Kularatne S, Handagala S, Ranasinghe G, Samarasinghe R. Ortner's syndrome presenting as thoracic aortic aneurysm mimicking thoracic malignancy: A case report. J Med Case Rep. 2015; 9:147.
73. Verbeke X, Vliebergh J, Sauer M, Leys M. Hoarseness revealing Ortner's syndrome. Acta Clin Belg. 2015; 70:230.
74. Zangirolami AC, Oliveira FV, Tepedino MS. Ortner's syndrome: Secondary laryngeal paralysis caused by a great thoracic aorta aneurysm. Int Arch Otorhinolaryngol. 2015; 19:180-182.
75. Akbulut S, Inan R, Demir MG, Cakan D. Laryngeal electromyography is helpful for cardiovocal syndrome. Acta Medica (Hradec Kralove). 2016; 59:29-32.
76. Al Kindi AH, Al Kindi FA, Al Abri QS, Al Kemyani NA. Ortner's syndrome: Cardiovocal syndrome caused by aortic arch pseudoaneurysm. J Saudi Heart Assoc. 2016; 28:266-269.

77. David JJ, Mohanlal S, Sankhe P, Ghildiyal R. Unilateral right pulmonary artery agenesis and congenital cystic adenomatoid malformation of the right lung with Ortner's syndrome. Lung India. 2016; 33:553-555.
78. De Freitas S, Connolly C, Neary C, Sultan S. Ductus arteriosus aneurysm presenting as hoarseness: successful repair with an endovascular approach. J Surg Case Rep. 2016; 2016.
79. Deveci OS, Celik AI, Cagliyan CE, Ozbarlas N, Demirtas M. Case images: Ortner's Syndrome caused by ductus arteriosus aneurysm. Turk Kardiyol Dern Ars. 2016; 44:352.
80. Martins RH, Pessin AB, Dias NH, Tunes R, Elias TG. Ortner's syndrome: A rare cause of sudden hoarseness in the older person. Age Ageing. 2016; 45:177.
81. Mesolella M, Ricciardiello F, Tafuri D, Varriale R, Testa D. Delayed recurrent nerve paralysis following post-traumatic aortic pseudoaneurysm. Open Med (Wars). 2016; 11:215-219.
82. Ogawa-Momohara M, Muro Y, Hirashiki A, Fujimoto Y, Kondo T, Akiyama M. Ortner's syndrome caused by pulmonary arterial hypertension associated with mixed connective tissue disease. Clin Exp Rheumatol. 2016; 34:1125.
83. Sarin V, Bhardwaj B. Ortner's syndrome-A rare cause of hoarseness: Its importance to an otorhinolaryngologist. Iran J Otorhinolaryngol. 2016; 28:163-167.
84. Wang JY, Chen H, Su X, Zhang ZP. Aortic dissection manifesting as dysphagia and hoarseness: Ortner's syndrome. Am J Emerg Med. 2016; 34:1185 e1181-1183.
85. Acharya MN, Bahrami T, Popov AF, Kishore Bhudia S. Rapid resolution of Ortner's syndrome with giant left atrium after double-valve replacement surgery. Interact Cardiovasc Thorac Surg. 2017; 25:663-664.
86. Klee K, Eick C, Witlandt R, Gawaz M, Didczuneit-Sandhop B. Unilateral recurrent nerve palsy and cardiovascular disease - Ortner's syndrome. J Cardiol Cases. 2017; 15:88-90.
87. Sarangi PK, Hui P, Sagar HS, Kisku DK, Mohanty J. Combined left recurrent laryngeal nerve and phrenic nerve palsy: A rare presentation of thoracic aortic aneurysm. J Clin Diagn Res. 2017; 11:TD01-TD02.
88. Alsheikh B, Aljohani O, Coufal NG. Paediatric pulmonary hypertension caused by an ACVRL1 mutation presenting as Ortner syndrome. Cardiol Young. 2018; 28:1475-1476.
89. Kyaw WA, Lim CY, Khalil MAM, Lim KC, Chong VH, Tan J. A fatal case of Ortner's syndrome and dysphagia aortica secondary to rapidly expanding mycotic thoracic aortic aneurysm in a chronic kidney disease patient. SAGE Open Med Case Rep. 2018; 6:2050313X18799247.
90. Sim MY, Lim YC, So SC, Zhang J, Teo DB. A Hoarse Warning: Ortner Syndrome. Am J Med. 2018; 131:1460-1462.
91. Karkowski G, Kielczewski S, Lelakowski J, Kuniewicz M. Ortner's syndrome after cryoballoon ablation. J Interv Card Electrophysiol. 2019; 54:309-310.
92. Lamas ES. Ortner's syndrome due to giant cell arteritis. Eur Heart J Case Rep. 2019; 3.
93. Zheng XZ, Chu YH, Tsai CS, Lin CY. Successful thoracic endovascular aortic repair in Ortner's syndrome. Ann Vasc Surg. 2019; 57:275 e279-275 e212.
94. Jeong JO, Park YS, Park JH. Ortner's syndrome discovered by a routine echocardiographic examination: A huge aneurysmal dilatation of the aortic arch as a cause of hoarseness. Korean Circ J. 2021; 51:379-381.
95. Tammirajua I, Radha Krishna T, Vittal Prasadc P, Jagadish Babud K. Ortner's Syndrome (Cardiovocal Hoarseness) – A rare entity in modern era. IHJ Cardiovascular Case Reports. 2018; 2:82-84.
96. Yuan SM. Ortner (cardio-vocal) syndrome: a collective review. *https://applications.emro.who.int/imemrf/Kuwait_Med_J/Kuwait_Med_J_2014_46_1_3_13.pdf* (accessd August 17, 2023)
97. Woodson GE, Kendrick B. Laryngeal paralysis as the presenting sign of aortic trauma. Arch Otolaryngol Head Neck Surg. 1989; 115:1100-1102.
98. Chan P, Huang JJ, Yang YJ. Left vocal cord palsy: an unusual presentation of a mycotic aneurysm of the aorta caused by Salmonella cholerasuis. Scand J Infect Dis. 1994; 26:219-221.
99. Harano M, Tanemoto K, Kuinose M, Kanaoka Y, Kagawa S, Yoshioka T. A case of chronic traumatic dissecting aneurysm of the thoracic aorta. Kyobu Geka. 1994; 47:1023-1025. (in Japanese)
100. el-Ahmady LM. Left vocal cord paralysis in bilharzial pulmonary aneurysm (case report). J Egypt Med Assoc. 1974; 57:232-236.
101. Wilmshurst PT, Webb-Peploe MM, Corker RJ. Left recurrent laryngeal nerve palsy associated with primary pulmonary hypertension and recurrent pulmonary embolism. Br Heart J. 1983; 49:141-143.
102. Kheok SW, Salkade PR, Bangaragiri A, Koh NSY, Chen RC. Cardiovascular Hoarseness (Ortner's Syndrome): A Pictorial Review. Curr Probl Diagn Radiol. 2021; 50:749-754.

Received June 3, 2023; Revised August 18, 2023; Accepted August 21, 2023.

*Address correspondence to:
Arunabh Talwar, Division of Pulmonary Medicine, North Shore University Hospital, Manhasset, NY 11030, USA.
E-mail: arunabh@northwell.edu

Released online in J-STAGE as advance publication August 23, 2023.

Review

DOI: 10.5582/irdr.2023.01052

Diagnosis, treatment, and research status of rare diseases related to birth defects

Hongjuan Zhao[1,§], Chen Du[2,§], Guang Yang[3,*], Yu Wang[2,*]

[1] Department of Gynecology and Obstetrics, Shandong Provincial Third Hospital, Shandong University, Ji'nan, China;
[2] Department of Gynecology and Obstetrics, Inner Mongolia Medical University Affiliated Hospital, Hohhot, China;
[3] Department of General Surgery, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China.

SUMMARY Rare diseases are diseases that occur at low prevalence, and most of them are chronic and serious diseases that are often life-threatening. Currently, there is no unified definition for rare diseases. The diagnosis, treatment, and research of rare diseases have become the focus of medicine and biopharmacology, as well as the breakthrough point of clinical and basic research. Birth defects are the hard-hit area of rare diseases and the frontiers of its research. Since most of these defects have a genetic basis, early screening and diagnosis have important scientific value and social significance for the prevention and control of such diseases. At present, there is no effective treatment for most rare diseases, but progress in prenatal diagnosis and screening can prevent the occurrence of diseases and help prevent and treat rare diseases. This article discusses the progress in genetic-related birth defects and rare diseases.

Keywords rare diseases, birth defects, diagnosis, treatment, prevention and control

1. Introduction

Rare disease is defined as a disease with a prevalence of less than 1/2,000 with a total number of patients less than 200,000 by The European Organization for Rare Diseases (EURORDIS) and the United States, respectively. The World Health Organization (WHO) defines a rare disease as one affecting between 0.065% and 0.1% of the population. It is estimated that there are 300 million rare disease patients worldwide, and more than 7,000 rare diseases account for 10% of all human diseases. Of these, 80% are inherited, and approximately 50% develop at birth or during childhood. Rare diseases often progress rapidly and have a high mortality rate; and less than 1% of rare diseases have specific drugs for treatment (*1*). An epidemiological survey in Ireland in 2020 showed that rare diseases were the main cause of death in children, accounting for 58.6% of the deaths in children aged 14 years and younger (*2*).

The medical and social needs of individuals with rare diseases are not met despite the large number of patients and families affected by them internationally. A general lack of public awareness and limitations in expertise have left rare disease patient populations overlooked and marginalized in healthcare systems and policies. EURORDIS, the International Society for Rare Diseases, and the NGO Committee for Rare Disease called for a UN resolution on Rare Diseases in 2019 and urged the 193 Member States of the UN General Assembly to adopt a resolution on December 16, 2021 (*3*). R&D in rare diseases is necessary to advance the UN commitment to achieve the 2030 Sustainable Development Goals of "leaving no one behind".

In recent years, many countries have carried out research on registering rare diseases as single or multiple diseases; 13,703 articles on this topic existed until April 2, 2023. The Orphan Drug Act of the United States in 1983 established the criteria for rare diseases, with other countries introducing similar criteria. On June 25, 2018, the First List of Rare Diseases was issued jointly by five institutions in China, including 121 common rare diseases that have become the standard for defining rare diseases in China (*4*).

Worldwide, serious birth defects occur in 3–6% of newborns, and on average, a child with a birth defect is born every 4.5 minutes. Due to the large population, rare diseases are not uncommon in China, and it is estimated that approximately 20 million people are affected. Genetic defects account for 71.9% of these rare diseases (*5*).According to WHO estimates, about a quarter of birth defects in the world are related to genetic factors. In the "First List of rare Diseases", a considerable number of metabolic and genetic diseases were included. The

research scope of rare diseases is also increasing, from neural tube malformation to congenital heart disease.

With the development of maternal-fetal medicine, fetal surgery, non-invasive prenatal testing, including cell-free fetal DNA, and next-generation sequencing technology (NGS), prenatal diagnosis and screening of these diseases are progressing, which provides help for the prevention and treatment of rare diseases.

2. Importance of rare diseases associated with birth defects

Birth defects and rare diseases, including structural abnormalities, functional abnormalities, and metabolic abnormalities, have become public health problems that affect life quality and community health. The related incidence are shown in Table 1.

2.1. Genetic factors associated with birth defects

According to the WHO, about a quarter of birth defects in the world are due to genetic factors. The "First List of Rare Diseases", published in 2018, includes a considerable number of metabolic and genetic diseases. Genetic etiology research is the basis for the prevention and treatment of birth defects. Genetic factors include mostly gene mutations and a small number of chromosomal abnormalities (*6*).

2.1.1. Monogenic genetic diseases and treatment strategies

Rare genetic diseases are usually monogenic (*7*), and include more than 6,000 types with complex clinical and genetic heterogeneity. Gene mutations include point mutation and dynamic mutation, as well as deletion or repetition of fragments and genes, which increase the difficulty in diagnosing such diseases (*8*). Currently, many monogenic diseases. Currently, many monogenic diseases such as cystic fibrosis, spinal muscular atrophy, thalassemia, Duchenne muscular dystrophy, hemophilia, osteogenesis imperfecta, and phenylketonuria are included on the list of rare diseases.

As far as diagnosis is concerned, monogenic genetic diseases involve many disciplines, and the clinical symptoms are complex. Because of the diversity of gene mutations, each mutation may lead to different clinical symptoms, and the clinical significance of mutations is quite complex, with high genetic heterogeneity and clinical heterogeneity, so clinical diagnosis is difficult. Nowadays, with the sharp drop in the cost of gene sequencing, the emergence of gene big data is followed. In this situation, it is an easy and effective way to establish an auxiliary diagnosis system and clinical knowledge base based on the relationship between genotype and phenotype.

Currently, there is a lack of effective treatments for most monogenic diseases. Mutated alleles revealed by sequencing with aneuploidy and linkage analyses (MARSALA) strategy for the combined diagnosis of monogenic and chromosomal diseases, along with preimplantation genetic testing (PGT) and NGS, play an important role in reducing the occurrence of birth defects and rare genetic diseases (*9*).

2.1.2. Polygenic genetic disease

Polygenic diseases have genetic heterogeneity but the same clinical phenotype, and there are several pathogenic genes. For example, deafness is associated

Table 1. Newborn incidence, incidence, and prevalence of rare diseases associated with birth defects

Diseases	No.	Disease	Newborn incidence / 100,000 persons	Incidence/ 100,000 persons	Prevalence/ 100,000 persons
Rare birth defects associated with structural abnormalities	1	Osteogenesis imperfecta (*4*)	7.14	/	0.089
	2	Crouzon syndrome (*28*)	16.5	/	/
	3	Saethre-Chotzen syndrome (*25*)	1	/	/
	4	Goldenhar syndrome (*20*)	/	3.79–17.86	/
	5	Beckwith-Wiedemann syndrome (*17*)	7.29–10	/	/
Rare birth defects associated with abnormal function	6	non-syndromic hearing loss (*30*)	186	/	/
	7	Retinoblastoma (*39*)	5–6.67	/	1-9
	8	Progressive muscular dystrophy (*4*)	16.67–27.78	25.30	/
	9	Leber hereditary optic neuropathy (*4*)	/	/	1.092
	10	Mitochondrial encephalomyopathy	/	/	/
	11	Parkinson's disease (*4*)	/	/	7.39
	12	Branchio-oto-renal syndrome (*35*)	2.5	/	/
Metabolic abnormalities associated with rare birth defects	13	Phenylketonuria (*4*)	/	8.48	1.17
Others	14	Ocular albinism (*4*)	/	5.56	/
	15	Epidermolysis bullosa (*54*)	/	/	0.30
	16	Hemophilia (*4*)	/	/	2.73
	17	Stickler syndrome (*68*)	/	11.11–13.33	2.7

with as many as 400 genetic syndromes, corresponding to at least 140 gene loci (*8*). Most birth defects are the result of multifactorial gene-environment interactions. Robert *et al.* discussed the various models, with their strengths and weaknesses, on the etiology of multifactorial birth defects and compared them with other diseases caused by gene-environment interactions, including primary immunodeficiency and cancer (*10*). They proposed that multi-factor gene-environment interactions will be an important research direction in the future.

The combination of fifth-generation mobile networks (5G) and blockchain technology has provided a new direction for PGT to accurately control rare genetic diseases. Hefeng Huang's team (*11*) revealed for the first time the precise mechanism of epigenetic methylation in intergenerational transmission of exogenous diabetes. by analyzing the lack of insulin secretion in the offspring of individuals with TET3 deficiency. This provides new scientific perspective for understanding and preventing chronic diseases such as diabetes from childhood. The world's first Preimplantation Genetic Testing for Polygenic Disease (PGT-P) optimized low-risk diabetic test-tube baby was born in Shanghai on August 2, 2022. The construction idea of building a genetic diagnosis cloud service platform based on 5G+ blockchain, a remote collaborative diagnosis platform, and a China population genome mutation database based on the current development status of PGT in China allowed prevention and control of rare genetic diseases in China at the international frontier level.

The Chinese human phenotype ontology (CHPO) database launched in 2016 aims to establish standard Chinese clinical phenotype terminology to better guide clinicians and research on genetic diseases. The fifth update was completed on May 8, 2023 (*https://www.chinahpo.net/*), corresponding to HPO version V2022-10-05, with 16,691 entries after this update.

2.2. Rare birth defects associated with structural abnormalities

Structural abnormalities are usually manifested as changes in physical structure, such as osteogenesis imperfecta (OI), congenital achondroplasia, polycystic kidney disease, and congenital heart disease.

2.2.1. Osteogenesis imperfecta

OI, also known as fragile bone disease, is a monogenic hereditary bone disease with a prevalence of 1/15,000 to 1/20,000 (*12*). Based on phenotypic classification, OI can be classified into 18 subtypes (*13*). Clinically, autosomal recessive inheritance is rare, whereas autosomal dominant inheritance is most common. OI type I, caused by mutations in the *COL1A1/2* gene encoding collagen type I, accounts for 85–90% of the cases.

Currently, there is no standardized treatment for OI. In clinical practice, bisphosphonates are used to treat OI. Teriparatide and transforming growth factor-β monoclonal antibodies are expected to increase bone density (*14*). To date, only two haploinsufficient (HI) mouse models of mild OI, Col1a1 +/Mov13 (Mov13) and Col1a1 +/-365, have been established. To facilitate OI research and the testing of new therapies, Claeys *et al.* found that the expression of collagen and bone metabolism markers was significantly increased in Mov13 mice compared with WT, although there was no significant change in Col1a1 expression, bone structure, or strength (*15*). However, in adult Mov13 mice, bone phenotype variability and severe lymphoma appear. Therefore, new HI OI mouse models and long-term drug studies are required. In addition, a recent case report of an 11-year-old patient with OI showed that physical therapy rehabilitation plays an important role in the management of OI and in improving life quality (*16*).

2.2.2. Beckwith–Wiedemann syndrome (BWS)

BWS is a rare disease associated with the aberrant expression of an imprinted gene cluster located on chromosome 11, region 15.5. The incidence of live births is approximately 1/10,000–1/13,700. There was no ethnic specificity, and the male-to-female ratio was approximately 1:1 (*17*). Without intervention, children may die of difficulty breathing and eating, as well as hypoglycemia, electrolyte disturbances, or tumors. Therefore, early diagnosis and treatment are important for children with BWS. In 2018, the European Congenital Imprinting Disease Network formulated an expert consensus on the diagnosis and scoring of BWS (*18*). BWS is usually diagnosed after birth, specifically during the neonatal period; however, prenatal screening is recommended for those with a positive family history or BWS suggested on prenatal ultrasound. The prenatal ultrasound detection rate for BWS is 64.1%, and one-year survival rates were over 90% (*19*). Clinicians should strengthen their understanding of BWS and make timely diagnoses. BWS should be highly suspected in infants with special manifestations such as omphalocele, hypoglycemia, macroglossia, and excessive weight gain after birth. Genetic testing should be performed early to assist in diagnosis and identifying the molecular subtypes, which can help with subsequent treatment and prognosis.

2.2.3. Goldenhar syndrome (GS)

GS is a rare congenital malformation that manifests as abnormal organ system development originating from the first and second branchial arches. The prevalence of GS is approximately 1/5,600–1/26,370 (*20*), and is more common in males than in females. Due to the lack of clear diagnostic criteria and large-scale clinical

research, it is difficult to accurately calculate the prevalence of GS. The etiology of GS is complex and diverse, involving genetic and environmental factors, and research on the pathogenesis of GS is still in its infancy. GS is usually sporadic, although familial cases have occasionally been reported. In addition, mutations in chromosomes 1, 4, 5, 7, 6, 9, 10, 12, 14–18, 22, and X have been reported (*21*). Lopez *et al.* proved that *MYT1* is a candidate gene locus for GS by performing whole-exome sequencing and single nucleotide polymorphism sequence analysis (*22*). Tingaud-Sequeira *et al.* found a nonsense mutation in *ZYG11B* in a patient with GS after whole-exome sequencing and confirmed the role of this gene in craniofacial cartilage structure and notochord development in cell and animal models (*23*). They demonstrated the involvement of the *EYA3* gene in the occurrence of GS at the cellular and animal levels (*24*). In many cases, no significant chromosomal or genetic abnormalities have been identified. Most of candidate pathogenic genes are involved in the migration and differentiation of neural crest cells. *EPAS1* is closely related to the formation of blood vessels, providing the basis for the neural crest cell hypothesis. However, the specific mechanism must be verified with further studies.

Even though familial GS cases are rare, genetic counseling is important. With the improvement in prenatal diagnostic technologies, the severity of GS has decreased in recent years. The ocular manifestations of GS are diverse and require individualized treatment plans. Surgery is the primary treatment for ocular abnormalities. Considering the impact on visual development and mental health, intervention at a young age is recommended.

2.2.4. Saethre–Chotzen syndrome (SCS)

Craniosynostosis is a disease characterized by the premature closure of one or more cranial sutures, resulting in skull deformity and brain dysfunction. It occurs in one in 100,000 live births (*25*). Craniosynostosis can be divided into comprehensive and non-syndromic craniosynostosis-based anomalies in other organs such as the heart, limbs, and respiratory system. Non-syndromic craniosynostosis accounts for approximately 85% of all craniosynostoses and is sporadically present in approximately 92% of patients. Syndromic craniosynostosis accounts for approximately 15% of the total incidence of craniosynostosis, and the most common genetic causes are mutations in *FGFR2*, *FGFR3,* and *TWIST1* (*26*). Heterozygous *TWIST1* mutations cause Saethreo-Chotzen syndrome, which is characterized by hypertelorism, microtia, low-set ears, and a low hairline. The cranial and facial deformities vary, and require personalized treatment strategies (*27*). Current research mainly focuses on craniofacial plastic surgery and ophthalmic correction.

2.2.5. Crouzon syndrome (CS)

CS is a rare autosomal dominant genetic disorder characterized by craniosynostosis resulting in skull deformity, facial dysmorphism, the incidence in live births is about 1.65/100,000. CS is associated with various mutations in fibroblast growth factor receptor 2 (*FGFR2*). *FGFR2* and *FGFR3*-related craniosynostosis shows obvious mandibular morphological changes in the early stages, which confirms the hypothesis of a genotype-phenotype correlation related to mandibular morphology (*28*). CS is usually diagnosed prenatally or during delivery through clinical and physical evaluations, as well as various targeted laboratory tests. The diagnosis of craniostenosis craniostosis requires amniocentesis, chorionic villus sampling, or preimplantation diagnostic studies of *FGFR1*, *FGFR2*, and *FGFR3* mutations. Children with CS require multidisciplinary cooperation and long-term management from various specialties. The appearance of children with CS can significantly improve after a series of surgical and orthodontic procedures. Many studies on drug therapy for pathogenic genes, such as the role of tyrosine kinase inhibitors in mutated FGFR-related craniostenosis, exist; however, use of these in a clinical setting requires time (*29*).

2.3. Rare birth defects associated with abnormal function

2.3.1. Hearing impairment related diseases

Non-syndromic hearing loss (NSHL): Deafness is the most common sensory disorder, with an incidence of 1.86% in newborns worldwide. Currently, more than 60% of deafness cases are caused by genetic factors, of which approximately 70% are NSHL. Its clinical manifestation is auditory dysfunction without other organ or system abnormalities (*30*). Wang *et al.* studied the effectiveness of combined methods to screen newborns for hearing impairment, and the results showed that compared with physical screening, genetic testing could identify an additional 13% of missed patients and 0.23% of newborns carrying drug-induced deafness susceptibility gene variants (*31*).

Currently, 124 genes for hereditary non-syndromic hearing loss have been reported (*https://hereditaryhearingloss.org/*), among which *GJB2*, mitochondrial 12 s rRNA, *SLC26A4* deaf, and *GJB3* were reported in the Chinese population. In addition, 3299C>A (p.Ser1100Tyr) and 5185-2A>G were found in a family with non-syndromic hearing loss. Among these, 5185-2A>G is a newly discovered intronic variant of the *TRIOBP* gene and expands the spectrum of *TRIOBP* deaf-causing variants. In NSHL, race seems to play a role in determining the genetic burden of *LOXHD1*, NSHL is mainly diagnosed through tertiary prevention, neonatal hearing, and deafness gene screening. Most patients with NSHL present with congenital or delayed

sensorineural hearing loss. The treatment of NSHL mainly focuses on avoiding predisposing factors and cochlear implants. Several studies have demonstrated successful gene therapy in mouse models (*32,33*). Patient-derived induced pluripotent stem cells are also being investigated because of the unique properties of stem cells that are pluripotent and self-regenerating (*34*). Therefore, determining the etiology of NSHL will improve management and treatment strategies. Genetic variation data from different populations worldwide are expected to be available for genetic counseling and prenatal diagnosis.

Branchio-oto-renal syndrome (BOR): BOR is a rare autosomal dominant genetic disease with a neonatal incidence of 1/40,000, accounting for 2% of severely deaf children (*35*). Previous studies have shown that 88% of patients with BOR have hearing impairments; 73% have preauricular fistulas; 60% have branchial cleft cysts or fistulas and sinus tracts; and 10% have hypoplastic renal function (*36*). The *EYA1* (8q13.3) gene abnormality is the most common cause of BOR, with approximately 40% of patients carrying this gene mutation. Approximately 10% of the patients have *SIX1* (14q23.1) and *SIX5* (19q13.32) gene mutations (*37*). There are few reports on BOR in China. Approximately 50% of patients with BOR do not have pathogenic variants of *EYA1*, *SIX1*, or *SIX5*, which may be related to the existence of other unexplored regions and novel gene variants.

The low incidence and diverse clinical manifestations of BOR have brought great difficulties and challenges to its clinical diagnosis and treatment, especially prenatal diagnosis, which can easily lead to missed diagnoses. A case of BOR diagnosed prenatally due to renal dysplasia was detected by ultrasound at 33 weeks of gestation, at which point the pregnant woman finally chose induced labor (*38*). The BOR phenotype is highly heterogeneous and can cause severe deafness and a complete loss of renal function. Prenatal diagnosis can help families understand the fetal prognosis and make prudent pregnancy decisions.

2.3.2. Neonatal eye disease

Retinoblastoma (RB) is the most common primary intraocular malignant tumor in infants and young children and originates from primitive retinal stem cells or cone precursor cells. The incidence in newborns is 5-5.57/100,000, with 85% of cases occurring before the age of 3, and the prevalence rate is 1-9/100,000. With approximately 9,000 new cases worldwide each year, retinoblastoma is considered a curable cancer in high-income countries, with a disease-free survival rate of approximately 100% (*39*). However, the prognosis tends to be poorer in low- and middle-income countries, where more than 80% of global cases occur (*40*). RB can be classified into genetic and nongenetic types. Gene therapy has become a new therapeutic approach in recent years. Molecular and genetic studies have shown that species-specific intrinsic genetic redundancy and compensation among RB family members can prevent retinoblastoma in mice (*41*). Genetically engineered mouse models have provided important insights into RB biology, but there are differences between species, with the human tumor epigenome localized at a later stage of development than mouse tumors (*42*). Based on this, a human cancer model derived from induced pluripotent stem cells provides valuable insights into tumor cell origin and tumorigenesis following *RB1* inactivation (*43*). Individual differences exist in the diagnosis and treatment of RB.

2.3.3. Neuromuscular-related diseases

Progressive muscular dystrophy (PMD): PMD includes Duchenne and Becker muscular dystrophy (DMD/BMD). It is a common form of muscular dystrophy in childhood, and DMD is the most common disease with a poor prognosis. DMD/BMD is an X-linked, recessive, degenerative muscular disease. The incidence of DMD/BMD does not differ significantly between countries, regions, or races. The incidence of DMD/BMD is about 1/3,853 in China, and it is estimated that there are approximately 70,000 patients nationwide. PMD is caused by a dystrophin deficiency. The gene encoding dystrophin is located at Xp21.2 and contains 79 exons. Mutations in the dystrophin gene lead to the loss or disruption of its expression product, dystrophin (*44*). In addition to basic clinical manifestations and electromyography, the detection of DMD gene deletions or duplications has become an important diagnostic method. To date, there is no radical cure for DMD, and glucocorticoids remain the first choice of treatment. With a deeper understanding of DMD gene mutations, targeted therapy and targeted therapy for certain mutation sites are new directions for DMD treatment in the future.

Leber hereditary optic neuropathy (LHON): LHON is a maternally inherited optic nerve disease caused by mitochondrial DNA (mtDNA) mutations and is one of the most common blinding diseases in adolescents worldwide (*45*). The incidence of the disease is between 1/31,000 and 1/526,000, with racial differences. It generally manifests as painless vision loss in both eyes, successively or simultaneously, and the best-corrected visual acuity (BCVA) is usually below 0.1. The prognosis for visual acuity is poor, and a few patients show mild spontaneous visual improvement. At present, the treatment of this disease is limited, and early symptomatic, supportive, and drug treatment may improve the vision of patients (*46*). China's first ophthalmic gene therapy drug, NR082, has been officially approved by the State Medical Products Administration for clinical research on LHON treatment. Idebenone, a short-chain coenzyme Q10 analog, was

approved by the European Medicines Agency for the treatment of LHON in 2015. Several studies have shown that oral idebenone (900 mg/day) is helpful for the improvement of vision in LHON patients, especially in patients with the m.11778G>A mutation (*47*). Challenges such as drug dose, side effects, and the high price of gene therapy remain. Currently, gene therapy for LHON is only available for patients with the m.11778G>A mutation. Using the same principle, rAAV2-ND1 and rAAV2-ND6 can also be used to treat the other two primary mutations; however, their specific feasibility needs to be verified.

2.3.4. Mitochondria-related diseases

Mitochondrial encephalomyopathy (MELAS): MELA is a multisystem metabolic disease caused by mutations in mitochondrial DNA or nuclear DNA (*48*). It is usually caused by abnormal oxidative phosphorylation of the electron respiratory chain. MELAS is a hereditary disease, including maternal inheritance, autosomal inheritance, and X chromosome concomitant inheritance. In 2016, a cohort study of North-East England MELAS says the prevalence of the disease is 2.9/100,000 (nDNA), 9.6/100,000 (mtDNA). A total of 10.8/100,000 individuals carrying pathogenic mutations develop clinical symptoms. The prevalence of onset in children (<16 years of age) is 5-15/100,000 (*49*). Many types of MELAS exist, with diverse clinical manifestations, difficult diagnoses, high misdiagnosis rates, and second-generation sequencing. Currently, there is no specific treatment method, and the characteristics of multisystem damage make it difficult to implement gene or stem cell therapy. Clinical trials of empirical drug therapy should be combined with specific clinical manifestations and possible pathogeneses in patients.

Parkinson's disease (PD): Parkinson's disease (PD) is a complex neurodegenerative disorder. The onset age of < 50 years is defined as early onset parkinsonism (EOPD). The prevalence of young-onset Parkinson's disease (YOPD) between 21 and 50 years of age and juvenile Parkinsonism (JP) before 21 years of age is 7.39/100,000. EOPD is less common, accounting for 5–10% of all cases, approximately 5% in European and American countries, and 10% in Japan, and its incidence increases with age. Genetics may soon provide personalized predictions of heterogeneous characteristics that affect disease-related disabilities, such as dementia risk and PD subtypes. In addition to conventional physical and drug therapies, achievements have been made in the development of remission therapies and biomarker treatment strategies for PD. The most common and advanced genetically linked targets are α-synuclein (SNCA), leucine-rich repeat kinase 2 (LRRK2), and glucoseencephalosidase (GBA1) (*50*). The convergence of proteins at the lysosomal degradation system level provides further targets for new therapeutic candidates as well as for biomarker development, a key component of drug discovery efforts. PD is a complex, multifactorial disease for which precision medicine, personalized diagnosis, and targeted therapy are particularly suitable. Genetic testing should be combined with other biomarkers such as sleep or smell testing and neuroimaging to obtain useful predictive and/or diagnostic capabilities.

2.4. Metabolic abnormalities associated with rare birth defects

Rare birth defects related to metabolic disorders include phenylketonuria, congenital hypothyroidism, congenital adrenal hyperplasia, glucose-6-phosphate dehydrogenase deficiency, *etc*.; however, phenylketonuria (PKU), one of the most well-known diseases, is rare.

PKU is most commonly caused by an autosomal recessive defect in the phenylalanine hydroxylase (PAH) gene (OMIM 612,349), resulting in an elevated blood phenylalanine concentration. PKU is equally prevalent among men and women. The prevalence of PKU varies in different races and regions, and varies by race and geographic region. Approximately 1/24,000 people worldwide are affected by PKU. Its prevalence in China is 1/15,924, which is relatively high in Asia (*51*). Prenatal diagnosis of BH4 deficiency can also be made by evaluating the concentrations of biopterin and neopterin in the amniotic fluid.

In 2020, the Food and Drug Administration (FDA) granted an orphan drug designation to a candidate for the treatment of PKU, APR-OD031, a slow-release amino acid mixture edited with proprietary drug-delivery technology, to ensure physiological absorption of the delivered amino acids. In addition, gene therapy with daily subcutaneous injections of PEGylated Phe ammonialase is expected in recent clinical trials, and mRNA approaches are under investigation (*52*).

2.5. Other rare birth defects

2.5.1. Hereditary skin diseases

Ocular albinism (OA): The overall incidence of albinism worldwide is approximately 1/20,000, and the incidence in some African countries can be as high as 1 <B->5,000 (*53*). The overall incidence of albinism in the Chinese population is 1/18,000. OA is diagnosed based on clinical manifestations and routine auxiliary examinations, and genetic diagnosis is crucial for the treatment of patients with albinism.

Albinism has a high degree of genetic heterogeneity, and 20 genes have been found to be related to different clinical manifestations of albinism, such as GPR143, AROA, the pathogenic genes of ocular albinism in non-comprehensive albinism; The main pathogenic genes of oculocutaneous albinism (OCA) are TYR, P gene,

TYRP1, SLC45A2, OCA5, SLC24A5, and C10ORF11. LYST is the only pathogenic gene in the CHS of albinism syndrome, whereas there are up to 10 pathogenic genes in HPS, including HPS1, AP3B1, HPS3, HPS4, HPS5, HPS6, DTNBP1, BLOC1S3, BLOC1S6, and AP3D1. Albinism is a single-gene genetic disease caused mainly by point mutations, and there is currently no effective treatment. Surgery or drug treatment can be used to optimize vision, and regular preventive examinations or symptomatic treatments can be performed to detect skin tumors. For many people with albinism, social and psychological burdens, such as low self-esteem and relatively poor social life abilities, may be greater than medical problems and deserve more attention.

Epidermolysis bullosa (EB): EB is a group of genetic disorders leading to recurrent skin blistering, the most common cause of which is infection. The overall incidence and prevalence of EBV in the United States are approximately 20 cases per million live births and 11 cases per million people, respectively (*54*). Epidermolysis bullosa simplex (EBS) is the most common type of EB, and its specific mode of inheritance depends on the pathogenic variant. The EXPH5 or TGM5 mutation is an autosomal recessive inheritance, whereas the dominant negative missense mutation of the KRT5 or KRT14 gene is an autosomal dominant inheritance (*55*). EB is a skin disease, and most complications are skin-related. The most life-threatening complication is an infection. To date, there is no cure for EB, and optimal management must be multidisciplinary, involving wound care, pain control, infection control, nutritional support, and the prevention and treatment of complications. There are many new treatment methods for EB, such as gene editing, gene replacement (*56*), reverse mutation therapy (*57*), exon skipping (*58*), protein supplementation (*59*), read-through therapy (*60,61*) and some small-molecule drugs (*62*), *etc.* All the countries are actively exploring and conducting various preclinical and clinical tests. This provides new hope for better patient care.

2.5.2. Inherited blood diseases

Hemophilia is an X-chromosome-linked recessive hemorrhagic disease, mainly divided into hemophilia A and B, of which hemophilia A (HA) accounts for 80–85%. Hemophilia B (HB) accounts for 15–20%. The pathogenic genes F8 and F9 are located on Xq28 and Xq27.1, respectively. Based on the large number of mutations detected in thousands of hemophilia patients worldwide, the molecular basis of mutations in hemophilia is extremely diverse. The main mutation types of hemophilia A are inversion/recombination, point mutation, and insertion/deletion (*63,64*), and the main mutation types of hemophilia B are slight mutation, deletion/insertion (*65,66*). Hemophilia treatment is primarily based on replacement therapy, including on-demand and preventive treatments. Recently, gene therapy has achieved breakthroughs in both animal and clinical trials. A new generation of lentiviral vectors designed for the efficient delivery of transgenes to the liver offers the possibility of curing patients with hemophilia (*67*). PGT is an effective reproductive option for preventing the birth of children with hemophilia.

2.5.3 Stickler syndrome (SS)

Stickler syndrome is a rare inherited collagenous connective tissue disease mainly associated with mutations in the gene encoding collagen. The incidence rate is between 1/9,000 and 1/7,500 (*68*). The disease primarily involves ocular, joint, maxillofacial, and auditory abnormalities. Stickler syndrome can be divided into six types based on the presence of ocular abnormalities, vitreous phenotypes, and molecular genetic characteristics. Among them, type 1 is autosomal dominant inheritance, type 2 is autosomal recessive inheritance, and type 3 is autosomal recessive inheritance. They are caused by mutations in COL2A1, COL11A1, COL11A2, COL9A1, COL9A2, and COL9A3, respectively. Whole-exome sequencing (WES) detection covers the coding regions and exon-intron boundaries of approximately 20,000 genes and is of great value for the diagnosis of families with genetic diseases (*69*).

Genetic testing can improve the diagnostic rate of a disease and facilitate accurate typing. Prenatal diagnosis is the best strategy for avoiding birth. CMA, gene panel, or exome/genome sequencing can all be used for the genetic evaluation of Stickler syndrome, and genetic test results can be used to help guide treatment and management, help detect other high-risk individuals, and provide the risk of recurrence for the patient's offspring. Currently, there are few reports on the treatment of this disease, and variable case selection, lack of molecular genetic subtypes, and inconsistent treatment strategies have led to historical uncertainty regarding the safety and efficacy of preventive treatment.

3. Related technologies for diagnosis of rare diseases

With the progress of molecular genetics, molecular diagnosis technology, gene sequencing technology and genomics technology, the diagnosis of rare diseases has made great progress. For the diagnosis of rare diseases, although the traditional enzymatic detection technology still occupies an important position, it can no longer meet the demand. The rise of protein's genomics and metabonomics makes it possible to accurately diagnose a variety of rare diseases. At the same time, combining molecular imaging technology and bioinformatics technology, computer-aided diagnosis also shows a wide application prospect.

3.1. Classical gene detection methods

Classical genetic testing methods, such as karyotype analysis, fluorescence in situ hybridization (FISH), array comparative genomic hybridization (aCGH), multiplex ligation-dependent probe amplification (MLPA), *etc.*, are considered to be effective in detecting copy number variations of fragments > 400bp, which play an important role in the genetic diagnosis of rare diseases, but cannot detect a smaller range of gene mutations, and cannot detect pathogenic variations with constant copy number, such as balanced translocation or inversion.

3.2. Next-generation sequencing technology

The diagnostic test of NGS is not affected by genetic mode, and the sequencing results can not only provide us with the location information of pathogenic genes, but also provide important information such as the type of pathogenic mutations. In recent years, next-generation sequencing technology has rapidly risen with its accurate and efficient characteristics, and has become one of the important methods for auxiliary diagnosis of rare diseases. Next-generation sequencing technology can be divided into genome sequencing, transcriptome sequencing and chromatin immunoprecipitation-sequencing (ChIP-seq). Genome sequencing includes whole genome sequencing (WGS), WES, single-cell genome sequencing, amplicon sequencing, *etc.*

3.3. Genome Sequencing

The application of next-generation sequencing technology in the diagnosis of rare diseases can be divided into WGS, WES and targeted gene sequencing. Although exons only account for 1% of the whole genome, it is inferred that about 85% of pathogenic mutations occur in this region. WES obtains DNA sequence information of the whole genome exon region through next-generation sequencing technology, and combined with clinical phenotype and bioinformatics analysis, it can infer possible pathogenic genes. Compared with WGS, WES eliminates non-coding sequences in genes, requires relatively small sample size, and is more economical. Compared with targeted gene sequencing, WES does not need to predict pathogenic genes in advance, and is more suitable for patients with rare diseases that have difficulty in diagnosis, so it is favored by many clinicians.

In 2020, the American College of Medical Genetics and Genomics (ACMG) published guidelines for the application of fetal exome sequencing technology in prenatal diagnosis. It is pointed out that fetal exome sequencing technology is suitable for cases with abnormal ultrasound findings but negative karyotype and chromosomal microarray results (*70*). WES can effectively improve the detection rate and accuracy of prenatal diagnosis, and pathogenic genetic variations can be detected in approximately 10% of cases with negative gene chip results.

WGS uses NGS technology to obtain the sequence information of the whole genome, which is more effective than other gene sequencing technologies in identifying single base mutation, cleavage site mutation, intron mutation and multiple copy number mutation. Compared with WES, WGS has better consistency and less bias in measurement results; however, due to its high price, its clinical application is not as wide as WES.

Targeted target gene sequencing is a method of sequencing a specific target gene, which is an economically feasible method for the detection of single gene genetic diseases with known pathogenic genes. For example, Usher syndrome is a group of autosomal recessive single gene diseases. Despite the genetic heterogeneity, the method of targeted target gene sequencing can detect pathogenic mutations caused by different mutation modes such as single base or sequence rearrangement at a time, improving the diagnostic efficiency (*71*).

3.4. RNA sequencing

RNA sequencing can not only be used to diagnose some rare diseases involving specific RNA pathogenesis, but also to identify rare diseases caused by abnormal protein transcriptional levels caused by splice site variations. Abnormalities regulated by miRNA may be one look at the pathogenesis of rare diseases, and detection of the variation types of miRNA may provide a reliable basis for clinical diagnosis. The use of RNA sequencing to diagnose rare muscle diseases from a genetic perspective, and the detection of skeletal muscle sample RNA, indicates that RNA sequencing can effectively identify harmful splicing sites located in exon or deep intron regions, and the overall diagnostic rate can reach 35% (*72*). The application of RNA sequencing in peripheral blood RNA analysis can improve the clinical diagnostic rate, and reduce the interference of mutations of unknown significance in the diagnosis of rare diseases. Splice analysis combined with prediction software such as SpliceAI can determine the important splicing abnormalities at the diagnostic level, and clarify the functional effects of some mutations of unknown significance (*73*). In addition, by detecting disease-related miRNAs and tracing them to find the mRNAs and coding genes regulated by them, it is also helpful to study rare diseases with unknown pathogenic genes.

3.5. Third-generation sequencing technology (TGS)

TGS, namely single molecule sequencing, abandons the sample amplification step that is prone to error, realizes the sequencing of a single DNA molecule, and the measurement results can be exported immediately,

further shortening the sequencing time from several days to several hours or even several minutes, which is a faster and more efficient sequencing method than second generation sequencing. Merker *et al*. (*74*) used TGS to detect structural variation of the PRKAR1A gene, and thus diagnosed a case of Carney syndrome. Despite many advantages, TGS has a high error rate, SMRT can reach 15% (*75*). Although it is a random error that can be overcome by sequencing multiple times, it also faces problems in information storage and result interpretation and ethical issues related to genetic information.

3.6. Proteinomics

At present, the detection technology of proteinomics is mainly based on mass spectrometry and affinity-based protein analysis, which has shown outstanding advantages in the diagnosis of some rare diseases. For example, GM2 ganglioside deposition in neural stem cells of Tay-Sachs patients was successfully detected by liquid chromatography-tandem mass spectrometry (*76*). Guo *et al.* (*77*) established a method based on two-dimensional nanometer ultra-high performance liquid chromatography and mass spectrometry analysis, which can detect the down-regulation of frataxin protein level from platelets of Friedrich's ataxia patients, and this method can be used for auxiliary diagnosis of diseases or judgment of intervention effect. Protein level detection is an important part of the diagnosis of rare diseases, and the results of proteinomics analysis may be directly related to disease phenotype.

3.7. Metabonomics

Metabonomics is a method to systematically study the metabolites of small molecules (< 1,500 Da) produced by biochemical reactions, which can monitor cell pathways in real time and reflect the metabolic state of cells, tissues and even organs. The study of metabolites can also reveal the hidden biochemical mechanism of diseases and provide new ideas for the diagnosis of some rare genetic metabolic diseases. Graham *et al.* (*78*) evaluated and integrated WGS data and liquid chromatography-mass spectrometry non-targeted metabolomics data, and determined the variation and priority of congenital metabolic diseases, suggesting that metabolomics detection is of great significance for the screening and diagnosis of rare diseases, especially hereditary metabolic diseases.

3.8. Other auxiliary diagnosis methods

It is necessary to attach importance to the application of information technology in the diagnosis of rare diseases while developing various biological technologies for diagnosing rare diseases. Creutzfeldt-Jacob disease is characterized by high signal intensity in brain gyrus and striatum on FLAIR sequence on MRI, which has high diagnostic value and is listed as one of the diagnostic criteria of CJD. Serum ceruloplasmin < 80 mg/L is strong evidence for the diagnosis of Wilson's disease. SpliceAI software developed by Illumina Company in the United States can predict the location of genome splicing sites, and analyze high-throughput sequencing technical data to effectively identify non-coding gene mutations that can cause abnormal splicing events (*79*). Face2Gene system developed by American digital medical company can identify facial features related to diseases through photos, thus assisting in identifying rare diseases (*80*).

4. Prevention and control of birth defects and rare diseases in the era of genomic medicine

As mentioned above, in recent years, with the rapid development of genomic detection technologies such as high-throughput sequencing, the level of screening and diagnosis of hereditary birth defects and rare diseases has greatly improved, and genomics plays an important role.

4.1. PGT and preconception carrier screening to prevent birth defects: primary prevention and control

PGT can be used for molecular genetic screening and diagnosis of embryos or gametes, which involve fluorescence in situ hybridization, microarray analysis, and second-generation sequencing.

Indications for PGT-M include monogenic genetic diseases with clear pathogenic genes, such as achondroplasia, osteogenesis imperfecta, thalassemia, hemophilia, Duchenne muscular dystrophy, and hereditary polycystic kidneys. Continuous breakthroughs in new detection technologies have also opened new prospects for the prevention and control of rare genetic diseases. For example, single-cell whole-genome sequencing, including single sperm/egg whole-genome and transcriptome sequencing, can detect chromosomal abnormalities and base mutations in a single cell. Owing to its technical advantages of high accuracy and high genome coverage, it has been gradually applied in the field of reproductive medicine (*81*). The combination of single-cell genome amplification technology and deep sequencing will not only elucidate the pathogenesis of more genetic diseases but also facilitate high-throughput screening and diagnosis of birth defects and rare diseases.

4.2. Prenatal screening and diagnosis to reduce the birth of severe defects: secondary prevention and control

Noninvasive prenatal testing (NIPT) sequencing of fetal DNA in maternal blood has better sensitivity and specificity while avoiding unnecessary invasive prenatal examinations and has become an important means of screening for fetal aneuploidy diseases. The detection range of NIPT ranges from the early stage, which

mainly involves trisomy 21,18,13 to the screening of chromosomal microdeletion, microduplication, and other syndrome diseases (*82*). With the continuous development and application of sequencing methods and trophoblastic separation of fetal-derived cells, the sensitivity of NIPT in the detection of fetal sex chromosome abnormalities, microdeletions, and microduplications has greatly improved, and the detection time window has advanced, allowing sufficient time for clinical diagnosis and decision-making.

Genomic abnormalities, including chromosome numerical abnormalities, large duplications, and pathogenic copy number variations (pCNVs), are important causes of birth defects. Compared to other techniques such as karyotype analysis and chromosomal microarray analysis, CNV-seq has the advantages of a wide detection range, high throughput, simple operation, good compatibility, and low DNA sample volume. CNV-seq technology has been gradually applied in clinical practice as a first-line prenatal diagnostic tool.

4.3. Postpartum newborn screening to reduce the risk of disability: Three-level prevention and control

Neonatal disease screening refers to the mass screening of diseases with serious consequences in the neonatal period; early diagnosis and treatment can avoid or reduce harm to the greatest extent. Currently, newborn heel blood screening, tandem mass spectrometry screening, and newborn hearing screening are routinely performed. With the rapid development of gene sequencing technology, the prospects of using WES and WGS as new auxiliary methods have attracted considerable attention (*83*).

5. Conclusions

Prenatal diagnosis and maternal-fetal medicine, as "pioneers" in the diagnosis and treatment of rare diseases, play an extremely important role in the clinical and scientific research systems of rare diseases. Prenatal screening and diagnosis and their corresponding management, promotion, and establishment of norms have become "urgent matters". However, implementation and achievement of precision, safety, economy, and ethics are the difficulties that need to be overcome.

The development of genomic technology has advanced the diagnosis, prevention, and control of hereditary birth defects and rare diseases to new heights. The United Nations launched the 100,000 Genomes Project in 2013, which achieved great success and provided the basis for the National Health Service genomic medicine service, becoming the first national healthcare system to provide genomic medicine services at the WGS. To fully realize the great potential of genomics in scientific research and clinical practice, it is necessary to continuously update and improve interdisciplinary collaboration, cross-population, policies, and regulations to ensure a more comprehensive understanding of the pathogenesis of diseases and the correlation between phenotypes and genotypes, which will help improve the equity of global access and the return to genomics. To promote the establishment of new, more standardized, and individualized prevention and treatment strategies to block the occurrence of the disease from the root to the greatest extent and benefit more patients.

Funding: This work was supported by a grant from Shandong province traditional Chinese medicine science and technology project(2021Q127), Inner Mongolia Grassland Talents Innovation Team Project (2022), Translational medicine project of Inner Mongolia Medical University, Inner Mongolia Autonomous Region Science and Technology Plan Project (2022YFSH0067).

Conflict of Interest: The authors have no conflicts of interest to disclose.

References

1. Luzzatto L, Hyry HI, Schieppati A, Costa E, Simoens S, Schaefer F, Roos JCP, Merlini G, Kaariainen H, Garattini S, Hollak CE, Remuzzi G, Second Workshop on Orphan Drugs p. Outrageous prices of orphan drugs: A call for collaboration. Lancet. 2018; 392:791-794.
2. Gunne E, McGarvey C, Hamilton K, Treacy E, Lambert DM, Lynch SA. A retrospective review of the contribution of rare diseases to paediatric mortality in Ireland. Orphanet J Rare Dis. 2020; 15:311.
3. Rare Diseases International. UN resolution on persons living with a rare disease. *https://www.rarediseasesinternational.org/un-resolution/* (accessed May 20, 2023).
4. He J, Tang M, Zhang X, Chen D, Kang Q, Yang Y, Hu J, Jin C, Song P. Incidence and prevalence of 121 rare diseases in China: Current status and challenges. Intractable Rare Dis Res. 2019; 8:89-97.
5. Nguengang Wakap S, Lambert DM, Olry A, Rodwell C, Gueydan C, Lanneau V, Murphy D, Le Cam Y, Rath A. Estimating cumulative point prevalence of rare diseases: analysis of the Orphanet database. Eur J Hum Genet. 2020; 28:165-173.
6. MacLeod SM. Pediatric Drug Development: Concepts and Applications. Clin Pharmacol Ther. 2009; 86:583-584.
7. Deciphering Developmental Disorders S. Large-scale discovery of novel genetic causes of developmental disorders. Nature. 2015; 519:223-228.
8. Alford RL. Nonsyndromic hereditary hearing loss. Adv Otorhinolaryngol. 2011; 70:37-42.
9. Chen S, Yin X, Zhang S, *et al*. Comprehensive preimplantation genetic testing by massively parallel sequencing. Hum Reprod. 2021; 36:236-247.
10. Lipinski RJ, Krauss RS. Gene-environment interactions in birth defect etiology: Challenges and opportunities. Curr Top Dev Biol. 2023; 152:1-30.
11. Chen B, Du YR, Zhu H, *et al.* Maternal inheritance of glucose intolerance *via* oocyte TET3 insufficiency. Nature. 2022; 605:761-766.

12. Sam JE, Dharmalingam M. Osteogenesis Imperfecta. Indian J Endocrinol Metab. 2017; 21:903-908.
13. Marini JC, Forlino A, Bachinger HP, Bishop NJ, Byers PH, Paepe A, Fassier F, Fratzl-Zelman N, Kozloff KM, Krakow D, Montpetit K, Semler O. Osteogenesis imperfecta. Nat Rev Dis Primers. 2017; 3:17052.
14. Franzone JM, Shah SA, Wallace MJ, Kruse RW. Osteogenesis Imperfecta: A Pediatric Orthopedic Perspective. Orthop Clin North Am. 2019; 50:193-209.
15. Claeys L, Zhytnik L, Wisse LE, van Essen HW, Eekhoff EMW, Pals G, Bravenboer N, Micha D. Exploration of the skeletal phenotype of the Col1a1 (+/Mov13) mouse model for haploinsufficient osteogenesis imperfecta type 1. Front Endocrinol (Lausanne). 2023; 14:1145125.
16. Nangliya RM, Jain DS, Saklecha AV, Patil DS. Effect of physiotherapy rehabilitation on osteogenesis imperfecta with a midshaft tibial fracture in the 11-year-old patient: A case report. Pan Afr Med J. 2022; 43:201.
17. Cammarata-Scalisi F, Avendano A, Stock F, Callea M, Sparago A, Riccio A. Beckwith-Wiedemann syndrome: Clinical and etiopathogenic aspects of a model genomic imprinting entity. Arch Argent Pediatr. 2018; 116:368-373.
18. Brioude F, Kalish JM, Mussa A, *et al.* Expert consensus document: Clinical and molecular diagnosis, screening and management of Beckwith-Wiedemann syndrome: an international consensus statement. Nat Rev Endocrinol. 2018; 14:229-249.
19. Barisic I, Boban L, Akhmedzhanova D, Bergman JEH, Cavero-Carbonell C, Grinfelde I, Materna-Kiryluk A, Latos-Bielenska A, Randrianaivo H, Zymak-Zakutnya N, Sansovic I, Lanzoni M, Morris JK. Beckwith Wiedemann syndrome: A population-based study on prevalence, prenatal diagnosis, associated anomalies and survival in Europe. Eur J Med Genet. 2018; 61:499-507.
20. Barisic I, Odak L, Loane M, *et al.* Prevalence, prenatal diagnosis and clinical features of oculo-auriculo-vertebral spectrum: A registry-based study in Europe. Eur J Hum Genet. 2014; 22:1026-1033.
21. Beleza-Meireles A, Clayton-Smith J, Saraiva JM, Tassabehji M. Oculo-auriculo-vertebral spectrum: A review of the literature and genetic update. J Med Genet. 2014; 51:635-645.
22. Lopez E, Berenguer M, Tingaud-Sequeira A, Marlin S, Toutain A, Denoyelle F, Picard A, Charron S, Mathieu G, de Belvalet H, Arveiler B, Babin PJ, Lacombe D, Rooryck C. Mutations in MYT1, encoding the myelin transcription factor 1, are a rare cause of OAVS. J Med Genet. 2016; 53:752-760.
23. Tingaud-Sequeira A, Trimouille A, Marlin S, Lopez E, Berenguer M, Gherbi S, Arveiler B, Lacombe D, Rooryck C. Functional and genetic analyses of ZYG11B provide evidences for its involvement in OAVS. Mol Genet Genomic Med. 2020; 8:e1375.
24. Tingaud-Sequeira A, Trimouille A, Salaria M, Stapleton R, Claverol S, Plaisant C, Bonneu M, Lopez E, Arveiler B, Lacombe D, Rooryck C. A recurrent missense variant in EYA3 gene is associated with oculo-auriculo-vertebral spectrum. Hum Genet. 2021; 140:933-944.
25. Cornelissen M, Ottelander B, Rizopoulos D, van der Hulst R, Mink van der Molen A, van der Horst C, Delye H, van Veelen ML, Bonsel G, Mathijssen I. Increase of prevalence of craniosynostosis. J Craniomaxillofac Surg. 2016; 44:1273-1279.
26. Johnson D, Wilkie AO. Craniosynostosis. Eur J Hum Genet. 2011; 19:369-376.
27. Abulezz TA, Allam KA, Wan DC, Lee JC, Kawamoto HK. Saethre-Chotzen syndrome: A report of 7 patients and review of the literature. Ann Plast Surg. 2020; 85:251-255.
28. Morice A, Cornette R, Giudice A, Collet C, Paternoster G, Arnaud E, Galliani E, Picard A, Legeai-Mallet L, Khonsari RH. Early mandibular morphological differences in patients with FGFR2 and FGFR3-related syndromic craniosynostoses: A 3D comparative study. Bone. 2020; 141:115600.
29. Carter EP, Fearon AE, Grose RP. Careless talk costs lives: fibroblast growth factor receptor signalling and the consequences of pathway malfunction. Trends Cell Biol. 2015; 25:221-233.
30. Zhou Y, Li C, Li M, Zhao Z, Tian S, Xia H, Liu P, Han Y, Ren R, Chen J, Jia C, Guo W. Mutation analysis of common deafness genes among 1,201 patients with non-syndromic hearing loss in Shanxi Province. Mol Genet Genomic Med. 2019; 7:e537.
31. Wang Q, Xiang J, Sun J, *et al.* Nationwide population genetic screening improves outcomes of newborn screening for hearing loss in China. Genet Med. 2019; 21:2231-2238.
32. Omichi R, Shibata SB, Morton CC, Smith RJH. Gene therapy for hearing loss. Hum Mol Genet. 2019; 28:R65-R79.
33. Chen JR, Tang ZH, Zheng J, *et al*. Effects of genetic correction on the differentiation of hair cell-like cells from iPSCs with MYO15A mutation. Cell Death Differ. 2016; 23:1347-1357.
34. Romito A, Cobellis G. Pluripotent stem cells: Current understanding and future directions. Stem Cells Int. 2016; 2016:9451492.
35. Fraser FC, Sproule JR, Halal F. Frequency of the branchio-oto-renal (BOR) syndrome in children with profound hearing loss. Am J Med Genet. 1980; 7:341-349.
36. Morisada N, Nozu K, Iijima K. Branchio-oto-renal syndrome: Comprehensive review based on nationwide surveillance in Japan. Pediatr Int. 2014; 56:309-314.
37. Krug P, Moriniere V, Marlin S, Koubi V, Gabriel HD, Colin E, Bonneau D, Salomon R, Antignac C, Heidet L. Mutation screening of the EYA1, SIX1, and SIX5 genes in a large cohort of patients harboring branchio-oto-renal syndrome calls into question the pathogenic role of SIX5 mutations. Hum Mutat. 2011; 32:183-190.
38. Tang P, Li J, Li J, Yang J, Zhu J. Prenatal diagnosis and genetic analysis of a fetus with Branchio-oto-renal syndrome: A case report. Medicine (Baltimore). 2022; 101:e31172.
39. Global Retinoblastoma Study G, Fabian ID, Abdallah E, *et al.* Global Retinoblastoma Presentation and Analysis by National Income Level. JAMA Oncol. 2020; 6:685-695.
40. Chawla B, Hasan F, Azad R, Seth R, Upadhyay AD, Pathy S, Pandey RM. Clinical presentation and survival of retinoblastoma in Indian children. Br J Ophthalmol. 2016; 100:172-178.
41. McEvoy J, Flores-Otero J, Zhang J, *et al.* Coexpression of normally incompatible developmental pathways in retinoblastoma genesis. Cancer Cell. 2011; 20:260-275.
42. Aldiri I, Xu B, Wang L, *et al.* The dynamic epigenetic landscape of the retina during development, reprogramming, and tumorigenesis. Neuron. 2017; 94:550-568 e510.
43. Norrie JL, Nityanandam A, Lai K, *et al.* Retinoblastoma from human stem cell-derived retinal organoids. Nat

Commun. 2021; 12:4535.
44. Cordova G, Negroni E, Cabello-Verrugio C, Mouly V, Trollet C. Combined therapies for duchenne muscular dystrophy to optimize treatment efficacy. Front Genet. 2018; 9:114.
45. Yang S, Ma SQ, Wan X, He H, Pei H, Zhao MJ, Chen C, Wang DW, Dong XY, Yuan JJ, Li B. Long-term outcomes of gene therapy for the treatment of Leber's hereditary optic neuropathy. EBioMedicine. 2016; 10:258-268.
46. Zuccarelli M, Vella-Szijj J, Serracino-Inglott A, Borg JJ. Treatment of Leber's hereditary optic neuropathy: An overview of recent developments. Eur J Ophthalmol. 2020; 30:1220-1227.
47. Pemp B, Mitsch C, Kircher K, Reitner A. Changes in visual function and correlations with inner retinal structure in acute and chronic Leber's hereditary optic neuropathy patients after treatment with idebenone. J Clin Med. 2021; 10:151.
48. Zhang Z, Zhao D, Zhang X, Xiong H, Bao X, Yuan Y, Wang Z. Survival analysis of a cohort of Chinese patients with mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS) based on clinical features. J Neurol Sci. 2018; 385:151-155.
49. Gorman GS, Chinnery PF, DiMauro S, Hirano M, Koga Y, McFarland R, Suomalainen A, Thorburn DR, Zeviani M, Turnbull DM. Mitochondrial diseases. Nat Rev Dis Primers. 2016; 2:16080.
50. Polissidis A, Petropoulou-Vathi L, Nakos-Bimpos M, Rideout HJ. The Future of Targeted Gene-Based Treatment Strategies and Biomarkers in Parkinson's Disease. Biomolecules. 2020; 10:912.
51. Xiang L, Tao J, Deng K, Li X, Li Q, Yuan X, Liang J, Yu E, Wang M, Wang H, Liu H, Zhu J. Phenylketonuria incidence in China between 2013 and 2017 based on data from the Chinese newborn screening information system: a descriptive study. BMJ Open. 2019; 9:e031474.
52. Elhawary NA, AlJahdali IA, Abumansour IS, Elhawary EN, Gaboon N, Dandini M, Madkhali A, Alosaimi W, Alzahrani A, Aljohani F, Melibary EM, Kensara OA. Genetic etiology and clinical challenges of phenylketonuria. Hum Genomics. 2022; 16:22.
53. Marcon CR, Maia M. Albinism: Epidemiology, genetics, cutaneous characterization, psychosocial factors. An Bras Dermatol. 2019; 94:503-520.
54. Fine JD. Epidemiology of inherited epidermolysis bullosa based on incidence and prevalence estimates from the National Epidermolysis Bullosa Registry. JAMA Dermatol. 2016; 152:1231-1238.
55. Chong SC, Hon KL, Scaglia F, Chow CM, Fu YM, Chiu TW, Leung AKC. Severe generalized epidermolysis bullosa simplex in two Hong Kong children due to de novo variants in KRT14 and KRT5. Case Rep Pediatr. 2020; 2020:4206348.
56. Gaucher S, Lwin SM, Titeux M, Abdul-Wahab A, Pironon N, Izmiryan A, Miskinyte S, Ganier C, Duchatelet S, Mellerio JE, Bourrat E, McGrath JA, Hovnanian A. EBGene trial: patient preselection outcomes for the European GENEGRAFT ex vivo phase I/II gene therapy trial for recessive dystrophic epidermolysis bullosa. Br J Dermatol. 2020; 182:794-797.
57. Twaroski K, Eide C, Riddle MJ, Xia L, Lees CJ, Chen W, Mathews W, Keene DR, McGrath JA, Tolar J. Revertant mosaic fibroblasts in recessive dystrophic epidermolysis bullosa. Br J Dermatol. 2019; 181:1247-1253.
58. Bremer J, van der Heijden EH, Eichhorn DS, Meijer R, Lemmink HH, Scheffer H, Sinke RJ, Jonkman MF, Pasmooij AMG, Van den Akker PC. Natural exon skipping sets the stage for exon skipping as therapy for dystrophic epidermolysis bullosa. Mol Ther Nucleic Acids. 2019; 18:465-475.
59. South AP, Uitto J. Type VII collagen replacement therapy in recessive dystrophic epidermolysis bullosa-How much, how often? J Invest Dermatol. 2016; 136:1079-1081.
60. Kocher T, Koller U. Advances in gene editing strategies for epidermolysis bullosa. Prog Mol Biol Transl Sci. 2021; 182:81-109.
61. Kwong A, Cogan J, Hou Y, Antaya R, Hao M, Kim G, Lincoln V, Chen Q, Woodley DT, Chen M. Gentamicin induces laminin 332 and improves wound healing in junctional epidermolysis bullosa patients with nonsense mutations. Mol Ther. 2020; 28:1327-1338.
62. Atanasova VS, Russell RJ, Webster TG, Cao Q, Agarwal P, Lim YZ, Krishnan S, Fuentes I, Guttmann-Gruber C, McGrath JA, Salas-Alanis JC, Fertala A, South AP. Thrombospondin-1 is a major activator of TGF-beta signaling in recessive dystrophic epidermolysis bullosa fibroblasts. J Invest Dermatol. 2019; 139:1497-1505 e1495.
63. Yi S, Zuo Y, Yu Q, Yang Q, Li M, Lan Y, Huang L, Zhang Q, Qin Z, Luo J. A novel splicing mutation in F8 causes various aberrant transcripts in a hemophilia A patient and identifies a new transcript from healthy individuals. Blood Coagul Fibrinolysis. 2020; 31:506-510.
64. Atik T, Isik E, Onay H, *et al.* Factor 8 gene mutation spectrum of 270 patients with hemophilia A: Identification of 36 novel mutations. Turk J Haematol. 2020; 37:145-153.
65. Huang L, Li L, Lin S, Chen J, Li K, Fan D, Jin W, Li Y, Yang X, Xiong Y, Li F, Yang X, Li M, Li Q. Molecular analysis of 76 Chinese hemophilia B pedigrees and the identification of 10 novel mutations. Mol Genet Genomic Med. 2020; 8:e1482.
66. Lu Z, Zhang H, Chen C, Wu W, Wei H. The novel mutation p.Asp315Tyr causes severe hemophilia B by impairing coagulation factor IX expression. Thromb Res. 2021; 198:23-25.
67. Milani M, Annoni A, Moalli F, *et al.* Phagocytosis-shielded lentiviral vectors improve liver gene therapy in nonhuman primates. Sci Transl Med. 2019; 11:eaav7325.
68. Hoornaert KP, Vereecke I, Dewinter C, *et al.* Stickler syndrome caused by COL2A1 mutations: Genotype-phenotype correlation in a series of 100 patients. Eur J Hum Genet. 2010; 18:872-880.
69. Jelin AC, Vora N. Whole exome sequencing: Applications in prenatal genetics. Obstet Gynecol Clin North Am. 2018; 45:69-81.
70. Monaghan KG, Leach NT, Pekarek D, Prasad P, Rose NC, Practice AP, Guidelines C. The use of fetal exome sequencing in prenatal diagnosis: A points to consider document of the American College of Medical Genetics and Genomics (ACMG). Genet Med. 2020; 22:675-680.
71. Aparisi MJ, Aller E, Fuster-Garcia C, Garcia-Garcia G, Rodrigo R, Vazquez-Manrique RP, Blanco-Kelly F, Ayuso C, Roux AF, Jaijo T, Millan JM. Targeted next generation sequencing for molecular diagnosis of Usher syndrome. Orphanet J Rare Dis. 2014; 9:168.
72. Cummings BB, Marshall JL, Tukiainen T, *et al.* Improving genetic diagnosis in Mendelian disease with transcriptome sequencing. Sci Transl Med. 2017; 9:eaal5209.
73. Wai HA, Lord J, Lyon M, *et al.* Blood RNA analysis can

increase clinical diagnostic rate and resolve variants of uncertain significance. Genet Med. 2020; 22:1005-1014.
74. Merker JD, Wenger AM, Sneddon T, *et al.* Long-read genome sequencing identifies causal structural variation in a Mendelian disease. Genet Med. 2018; 20:159-163.
75. Suzuki Y, Wang Y, Au KF, Morishita S. A statistical method for observing personal diploid methylomes and transcriptomes with single-molecule real-time sequencing. Genes (Basel). 2018; 9:460.
76. Vu M, Li R, Baskfield A, *et al.* Neural stem cells for disease modeling and evaluation of therapeutics for Tay-Sachs disease. Orphanet J Rare Dis. 2018; 13:152.
77. Guo L, Wang Q, Weng L, Hauser LA, Strawser CJ, Rocha AG, Dancis A, Mesaros C, Lynch DR, Blair IA. Liquid chromatography-high resolution mass spectrometry analysis of platelet frataxin as a protein biomarker for the rare disease Friedreich's ataxia. Anal Chem. 2018; 90:2216-2223.
78. Graham E, Lee J, Price M, Tarailo-Graovac M, Matthews A, Engelke U, Tang J, Kluijtmans LAJ, Wevers RA, Wasserman WW, van Karnebeek CDM, Mostafavi S. Integration of genomics and metabolomics for prioritization of rare disease variants: A 2018 literature review. J Inherit Metab Dis. 2018; 41:435-445.
79. Jaganathan K, Kyriazopoulou Panagiotopoulou S, McRae JF, *et al.* Predicting splicing from primary sequence with deep learning. Cell. 2019; 176:535-548.e524.
80. Gurovich Y, Hanani Y, Bar O, Nadav G, Fleischer N, Gelbman D, Basel-Salmon L, Krawitz PM, Kamphausen SB, Zenker M, Bird LM, Gripp KW. Identifying facial phenotypes of genetic disorders using deep learning. Nat Med. 2019; 25:60-64.
81. Yan L, Yang M, Guo H, *et al.* Single-cell RNA-Seq profiling of human preimplantation embryos and embryonic stem cells. Nat Struct Mol Biol. 2013; 20:1131-1139.
82. Yin L, Tang Y, Lu Q, Pan A, Shi M. Application value of NIPT for uncommon fetal chromosomal abnormalities. Mol Cytogenet. 2020; 13:39.
83. Adhikari AN, Gallagher RC, Wang Y, *et al.* The role of exome sequencing in newborn screening for inborn errors of metabolism. Nat Med. 2020; 26:1392-1397.

Received June 29, 2023; Revised August 17, 2023; Accepted August 22, 2023.

[§]These authors contributed equally to this work.

**Address correspondence to:*
Yu Wang, Department of Gynecology and Obstetrics, Inner Mongolia Medical University Affiliated Hospital, Tongdao North Street No. 1, Hohhot 010050, China.
E-mail: wuai1544 @163.com

Guang Yang, Department of General Surgery, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China.
E-mail: aguangsun@sina.com.cn

Released online in J-STAGE as advance publication August 25, 2023.

Review

DOI: 10.5582/irdr.2023.01005

Carrier screening programs for rare diseases in developed countries and the case of Turkey: A systematic review

Çağlar Fidan[1,*], Recep Akdur[1], Çiğdem Naz Ünver[2], Ömer Can Şahin[2], Aslı Begüm Alper[2], Ali Ayhan[3]

[1] Department of Public Health, Başkent University Faculty of Medicine, Ankara, Turkey;
[2] Başkent University Faculty of Medicine, Ankara, Turkey;
[3] Department of Obstetrics and Gynecology, Başkent University Faculty of Medicine, Ankara, Turkey.

SUMMARY Effective control of rare diseases requires health programs based on principles of protection and prevention. Carrier screening programs serve as preventive measures by identifying at-risk groups. This review examines the impact, implementation, advantages, and disadvantages of carrier screening, incorporating examples from ten countries: the United States, Canada, the United Kingdom, Israel, China, Australia, Italy, Germany, the Netherlands, and Turkey. Data on carrier screening and related policies were collected from July to November 2022 and presented in a tabular format using a coding system devised by the authors. Variability was observed in the diseases/disorders and populations screened, screening expenses, and government provision across the countries. The number of diseases/disorders examined, ranging from 3 to 47, was determined by committee guidelines, government resources, pilot studies, and national institute resources. Notably, carrier screening programs exhibited greater worldwide inconsistency compared to newborn screening programs. The comparative analysis of developed countries serves to guide emerging nations. To address inequalities at both local and global levels, there is a need to enhance the establishment, development, and implementation of carrier screening programs. Furthermore, cost analyses of screening should be conducted, and adequate funding should be allocated to countries. In conclusion, this review highlights the preventive potential of carrier screening for rare diseases and emphasizes the importance of improving carrier screening programs globally to achieve equitable healthcare outcomes.

Keywords rare disease prevention, carrier screening, preconception carrier screening, premarital screening, early diagnosis screening

1. Introduction

A rare disease is defined by the fact that it affects a small percentage of the population. However, there is no internationally recognized definition of rare diseases (*1,2*). In the European Union, rare diseases are classified as life-threatening or chronically debilitating diseases that affect fewer than 5 in 10,000 people. In the United States, rare diseases are recognized as diseases that affect fewer than 200,000 people, or 1 in 1500, and in Japan, fewer than 50,000 people, or 1 in 2,500 (*1-3*). The diagnosis and treatment of rare diseases, which are regarded as significant global health issues, are challenging and expensive (*2,4,5*).

2. The significance of health screenings in rare diseases

As with all diseases, in order to be successful in controlling rare diseases, the health program to be implemented must be based on the public health principles of protection and prevention. Preventive health services are classified into five groups/classes. These groups are named primordial, primary, secondary, tertiary, and quaternary prevention. In this context, the use of screening tests in a comprehensive rare disease control program is the most important tool or intervention. The primary goals of screening programs are as follows: *i*) Identifying at-risk individuals or carriers for screening, protecting them from the risk, and preventing disease onset (primary prevention); *ii*) Detecting and efficiently treating affected individuals at an early stage (at the asymptomatic/preclinical stage) (secondary prevention) (*2*).

The application of screenings in rare disease control

programs at this point has two purposes: *i*) identification of individuals who are carriers (autosomal recessive (AR) or heterozygous for a pathogenic or possible pathogenic variant in an X-linked disease; in other words, those who are at risk of having an affected offspring (primary prevention); and *ii*) ensuring that affected individuals are diagnosed at an early stage and receive the most appropriate and effective treatment (secondary prevention) (*2,6*) (Figure 1).

3. Who should be screened?

Over 40 years ago, Wilson and Jungner, on behalf of the World Health Organization (WHO), developed a gold standard criterion for the evaluation of population-based screening. The Wilson and Jungner principles are a guide for how governments make decisions about screening, but how they are put into practice varies around the world to fit the needs of local circumstances (*2,7*). The American College of Obstetricians and Gynecologists (ACOG) issued a committee opinion in 2017, with an expanded panel recommending screening for conditions with a carrier frequency above 1%. Guo *et al*. also found that screening only for conditions with carrier frequencies above 1%, corresponding to variants in just 40 genes in their study, would identify 76–97% of carrier pairs. Chokoshvili *et al*. compared 16 different suppliers of expanded carrier screening panels, and they discovered that only three diseases (Cystic Fibrosis (CF), Maple Syrup Urine Disease 1B and Niemann-Pick Disease) were found to be screened in common (*2,8*). Concerning the current status of carrier screening, the American College of Medical Genetics and Genomics (ACMG) published a practice resource in 2021 and proposed the establishment of a tier-based carrier screening system. This system is divided into four tiers, with Tier 1 screening focusing on screening for CF and Spinal Muscular Atrophy (SMA) regardless of ethnicity or population. Tier 2 and tier 3 screenings propose using carrier frequency as a way of selecting what to include in carrier screening in the general population. Tier 4, on the other hand, doesn't have a minimum frequency requirement, and the number of conditions that can be screened can be substantially expanded. The practice resource recommends Tier 3 carrier screening be offered to all patients who are pregnant or planning pregnancies, and Tier 4 screening be used when a family or personal medical history necessitates the test and when the pregnancy is the result of a known or potential consanguineous marriage (*2,6*) (Figure 2).

4. An approach to the current status of carrier screening policies in the selected countries

Diagram: COMPREHENSIVE CONTROL PROGRAM FOR RARE DISEASES

(DISEASES ONCET AND PROGNOSIS)

PRECLINIC PHASE		CLINIC PHASE	
PRIMORDIAL PREVENTION	**PRIMARY PREVENTION**	**SECONDARY PREVENTION**	**TERTIARY PREVENTION**
Evaluation of the social determinants	Determinations of the risk groups	Early diagnosis	Monitoring of patient and ongoing care
Legal regulations	Elimination of risk factors	Suitable treatments	Rehabilitation interventions
Making the infrastructure for health services	Premarital genetic screenings	Easy access to health services	Independency of the people and adequate income
Public education and raising the literacy level of the public	Prevent risk factors	Education of health personnel	Social participation
Raising the welfare level of the public	Prevent exposures	Easy-to-use healthcare facilities	

QUARTERNARY PREVENTION

Prevention the overmedicalization
Careful prescription/Attentive medical intervention/avoiding side effect
Patient safety/Health services safety
Avoiding fragmented and extreme specialization
Combination of palliative care and monitoring

Figure 1. Diagram illustrating comprehensive control program for rare diseases. This diagram is developed by Dr. Akdur (*2*).

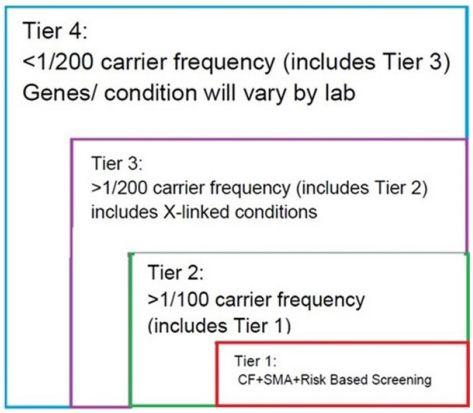


Figure 2. Diagram illustrating the tier based approach proposed by ACMG. This figure is based on the Tier Based Approach Proposed by ACMG (*6*).

In this review, databases, websites and organizations were examined with the purpose of examining the current literature on carrier screening. Additionally, the number of diseases/disorders screened by carrier screening in the United States, Canada, the United Kingdom, Israel, China, Australia, Italy, Germany, the Netherlands, and Turkey, the specific conditions under which these diseases/disorders are screened, and the official policies of these countries on carrier screening, including guidelines published by interested associations and organizations, were investigated. The official websites of the national health institutes and health ministries of these countries were analyzed.

4.1. The process of study selection and the strategy applied to obtain data

Databases such as PubMed, ResearchGate, and Google Scholar were searched with "((carrier screening [Title/Abstract]) or (carrier screening program [Title/Summary])) or ((preconception carrier screening [Title/Summary]) or (premarital screening [Title/Summary])" strategy with an emphasis on open access articles. "(("Country" carrier screening) or ("Country" carrier screening program))" search methods were adopted whilst websites, organizations and Google Search database, as well as the PubMed database, were studied. A total of 1,071 journal articles, books, and reports were scanned in the databases in addition to records from websites and organizations. Records published between 1972 and 2022 were examined, with an emphasis on the ones published in 2017 and later. The research began in October 2021 and was mainly conducted from July 2022 to November 2022. The study mostly utilized English-language sources, but it also examined documents in the national languages of Turkey, Italy and the Netherlands. The evaluation of the literature, data extraction and assessments were conducted collectively by the authors. The review was formatted based on the PRISMA guidelines (*9*).

4.2. Eligibility criteria and the assessment of risk of bias

The authors benefited from elimination criteria such as *i*) subject and terminology cooperation among obstetrics and gynecology and public health disciplines; *ii*) construction to the extent that it may benefit multiple specialties; *iii*) emphasis on reports written in English; and *iv*) attainment of coherence when the reports were analyzed for inclusion and exclusion. The risk of bias assessment was conducted with the ROBIS tool by the authors (*10*). Eligibility criteria, synthesis process, and findings were addressed. Additionally, the criteria for identifying and collecting the studies to be included in the paper were investigated.

5. Current status of carrier screening policies in selected countries based on the number of diseases/conditions screened and the spesific circumstances the diseases/conditions screened

A total of 32,662 records from 4 databases, 516 records from websites, and 464 records from organizations were identified with search engines. Subsequent to retrievals and assessments, 41 reports were included in the review (Figure 3)

In this review, the diseases/disorders screened for or recommended to be screened by the official carrier screening programs of the ten countries were described. The conditions under which these diseases were screened were included by searching publicly available sources on the internet, including databases, websites, and organizations. However, different types of resources have been publicly accessible for each country. Since there haven't been routinely created resources for rare diseases, the most recent and available materials were utilized in order to emphasize rare diseases. To address the issue, the documents were classified and prioritized according to this classification. The following documents were prioritized by the authors: government data, committee guideline data, pilot study data, and national institution data, respectively. The data obtained is presented as a table consisting of 73 diseases/disorders. A coding system was generated by the authors to demonstrate the data since the carrier screening plans of each country vary significantly. The coding system, consisting of numerals and letters, was explained in detail below the table. The codes "A, A1, A2, B, C, C1, C2, C3, C4, D, E" symbolize the conditions for which diseases are screened. The codes "cg, gd, ps, ni" are the abbreviations of the following document types: committee guideline data, government data, pilot study data, and national institution data, respectively. The codes "cg1, cg2, gd1, gd2, gd3, gd4, ps, ni1, ni2, x1" categorize and represent the data resources of each country (Table 1, Online Data, *http://www.irdrjournal.com/action/getSupplementalData.php?ID=165*).

The availability of different types of data

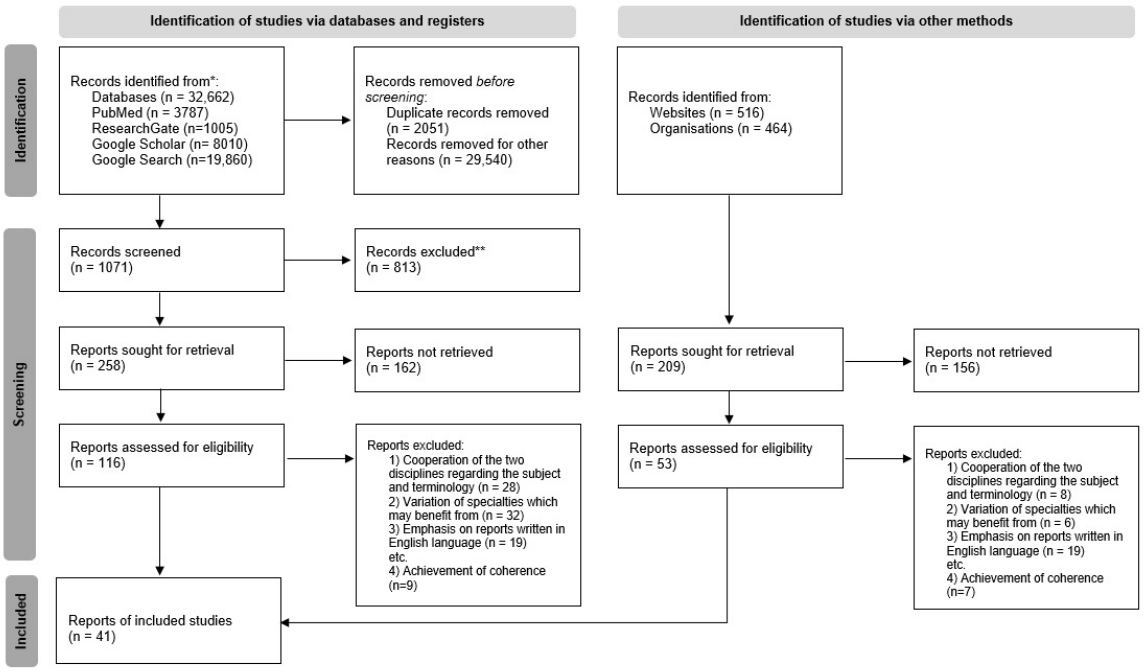


Figure 3. Diagram illustrating the identification of records. This diagram is developed based on PRISMA 2020 (*9*). The criterion of the two disciplines were formulated on obstetrics and gynecology and public health disciplines.

demonstrates the significance and implementation of carrier screening programs in various countries. However, a homogeneous comparison cannot be made between the carrier screening programs of the countries due to different data. In terms of application, government data and committee guideline data take precedence; however, it is not possible to rank the other forms of data.

The number of diseases/disorders screened by the ten countries ranged from 3 to hundreds. It is recommended to screen for 18 diseases/disorders in the USA (*11*) and 16 diseases/disorders in Canada (*12*) according to committee guidelines. 4 diseases/disorders in the UK (*8*), 30 diseases/disorders in Israel (*13,14*), and 3 diseases/disorders in Turkey (*15-17*) are screened for based on government resources. In Italy, 3 diseases/disorders are recommended to be screened by molecular genetic testing by the government resources (*18,19*). In the Netherlands, 47 diseases/disorders may be screened in a national health institute (*20*). In Australia, it is recommended to screen the general public for 3 diseases/disorders even though it is possible to screen for hundreds of diseases/disorders in a national health institute (*21-23*). 11 diseases were screened in a pilot study in China in 2019 (*24*). Although there are laboratories in Germany that are able to screen for numerous conditions, social screening is not currently available (*25*). Carrier screening programs were provided free of charge or for a fee by the government in some countries and for a fee by private health institutions in others. The carrier screening test is not covered by the government in the USA (*26*), Australia (*22*), Germany (*25*), or the Netherlands (*27*); however, private health insurance is accessible. Under specific conditions, such as being referred by a hospital specialist, having designated ethnic origins, and marriage, screening is covered by health insurance in the UK (*28*), Israel (*13*), and Turkey (*15,16*), respectively. While screening tests for some diseases/disorders were conducted nationwide, tests for other diseases/disorders were administered to at-risk groups identified by different countries based on their own populations (*8,11-18,20-24*). Compared to newborn screening (NBS), carrier screening programs revealed a higher degree of heterogeneity in terms of the diseases/disorders screened for, the number of diseases/disorders screened for, and the groups screened for, and the tests were generally not covered by the state or only a small number of diseases/disorders were covered by the state (*29*).

6. Carrier screening in Turkey

The carrier screening program in Turkey is available as the Premarital Screening Program, which consists of the Premarital Hemoglobinapathy Screening Program and the Premarital Carrier Screening Program for SMA.

6.1. Premarital screening program in Turkey

Anamnesis, physical examinations, and blood sampling for laboratory tests are administered to couples who are getting married. The program includes tests for hemoglobinopathy screening, blood group determination, SMA screening, and infectious disease screening (*16,30*).

6.2. Premarital hemoglobinopathy screening program in Turkey

The prevalence of beta-thalassemia carriers in the Turkish population is 2.1%, with regional variations ranging from 0.6% to 13%. In the absence of any intervention program, it is estimated that 400 new cases arise each year (*17*). Studies also revealed that Turkey has an alpha-thalassemia prevalence of 0.25%, with regional variations (*31*). Although the Law on Combating Inherited Blood Diseases was published in 1993 and the Regulation on Hemoglobinopathy Control Program and Diagnosis and Treatment Centers was published in 2002, thalassemia has not been eradicated in Turkey in 25 years. Thalassemia is still considered to be a condition that has a severe burden on the Turkish economy (*30*). The program aims to extend the life expectancy of existing hemoglobinopathy patients, increase their quality of life, and prevent abnormal hemoglobin diseases (*15*). The operation of Premarital Hemoglobinopathy Screening Program in Turkey was reported in Figure 4.

6.3. Premarital carrier screening program for SMA

Due to Turkey's significantly high carrier rates, the SMA carrier screening program began to be implemented in 81 provinces as of the end of December 2021. This initiative aims to identify couples who are both SMA carriers, give genetic counseling to families, inform and guide individuals about prenatal or pre-implantation diagnostic test choices, and reduce the long-term morbidity and mortality associated with SMA disease. SMA carrier screening is administered to spouses who apply for a premarital health evaluation and to married couples (*16*).

7. Importance of screening in consanguineous marriages

In clinical genetics, consanguineous marriages are defined as marriages between second cousins (fifth degree relatives) or more closely related family members (*32*). A study published in 1972 reported that the rate of consanguineous marriages in Turkey was 29.2%, and 80% of these marriages were between children

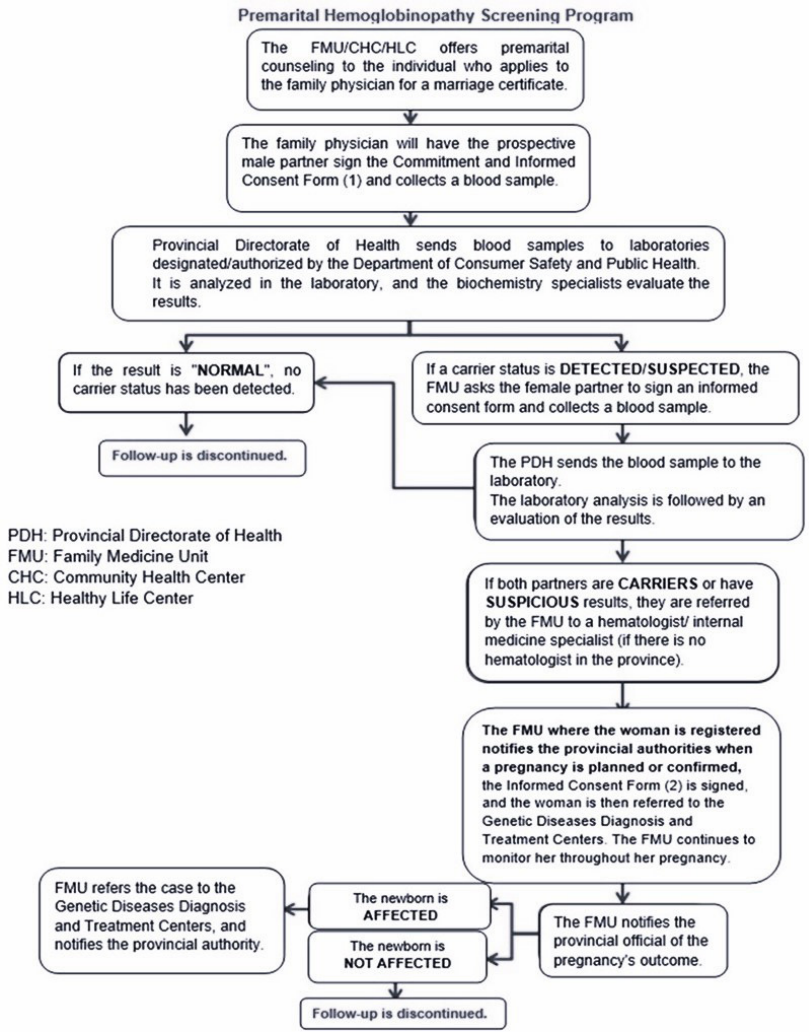


Figure 4. Diagram illustrating the operation of the Turkish premarital hemoglobinopathy screening program. This diagram is based on the Premarital Hemoglobinopathy Screening Program of the Republic of Turkey, Ministry of Health (*17*).

of siblings (*33*). Moreover, according to data from the Turkish Statistical Institute (TÜİK), 8.3% of the population were married to their first cousins in 2021 (*34*). The reproductive risk that sets consanguineous couples apart from other couples in the general population is reportedly related with AR diseases/ disorders, according to a 2021 study looking into the impacts of consanguineous marriages. A protocol based on whole exome analysis (WES) was developed, where the exomes of 39 consanguineous couples were studied, by the researchers in response to this statement. It was reported that eight couples shared heterozygosity for at least two pathogenic mutations, whereas 53.8% of couples shared heterozygosity for at least one variant that was thought to be pathogenic or possibly pathogenic for an AR disease. It was recommended that carrier screening with WES should be incorporated into genetic counseling for all consanguineous couples and since consanguineous couples occasionally have more than one shared pathogenic gene, even couples whose children have already been diagnosed with AR disease should undergo carrier screening with WES (*35*). In a different study, preconception carrier screening (PCS) was carried out on a population of Dutch women of Turkish and Moroccan descent in 2018, where the rate of consanguineous marriages in these groups was reported to be around 20–25%. Although already known to have a high incidence of AR diseases in their families, after the screening results were disclosed, they did not accept the reproductive options of prenatal genetic diagnosis (PND), pregnancy termination (TOP), *in vitro* fertilization (IVF) with donor oocytes, artificial insemination with sperm donation (AID), or adoption. IVF with preimplantation genetic diagnosis (PGD) was, on the other hand, widely accepted. The study also noted that women were vocal about avoiding getting married or even divorcing when both partners were carriers and favored PCS for premarital screening. In order to provide successful health care, it was claimed that bringing attention to consanguineous marriages and their effects, as well as being sensitive when giving information, screening, and counseling services to these families, were essential (*32*).

8. Advantages and disadvantages of carrier screening

The advantages of universal screening include the elimination of ethnic or racial factors, the reduction of stigma, and the removal of the burden on patients or physicians to recognize risk (*2,8*). For a screening program to remain as a beneficial source, however, long-term assessments must be made, and its application to the current context and conditions, its technological applicability, and the efficacy of the treatment that follows must also be taken into account. For instance, the impact of a nationwide CF carrier screening program in Israel reduced the number of infants born with CF who have a relatively severe phenotype. As a result, the program's organizers chose to remove CF from the NBS panel. When offered during the preconception period, carrier screening allows couples to make informed reproductive decisions, such as not having children, adopting, using PGD or IVF to avoid having an affected child, or having a child naturally while being aware of the risks. By providing prospective parents with a diagnosis before the baby is born, pre-conception screening can prevent the birth of an affected child. Attempting to prevent the decision to terminate an affected pregnancy makes it more favorable in this regard than prenatal screening (*2,7*).

Universal screening is expected to increase costs and complicate genetic variant analysis across laboratories. There will be a need to ensure that carrier screening tests have adequate accuracy and sensitivity across the population (*2,8*). The expansion of carrier screening panels, in contrast to NBS public health programs, is currently primarily driven by commercial interests, is not founded on professional guidelines or defined criteria, and leads to a wide selection of tests covering hundreds of conditions. It may appear appealing to use a single test for multiple diseases/disorders at nearly the same cost. However, Next Generation Sequencing (NGS) may inadvertently begin to include diseases/disorders that are mostly symptomatic in adults, less pathogenic DNA variants or variants of unknown significance. Also, not all prospective parents choose to participate in carrier screening tests or act on screening results because screening tests are not always able to detect all carriers. ACOG has stated that NBS remains significant as a screening method and cannot be substituted for this reason (*2,36*).

9. Public perspectives

In recent years, numerous studies have been conducted on diverse populations throughout the world with the objective of assessing public readiness and perspective on the subject of carrier screening, with a focus on issues including test providers, availability, suitable planning, and disclosure of the results, follow-up care, the hesitations and willingness of the people, and in what forms they should be expected to comply with these programs.

In 2018, Mathijssen *et al.* examined 182 participants' pre- and post-carrier screening experiences with the PCS offer for 4 AR illnesses in the Netherlands. It was mentioned that genetic conditions were publicly acknowledged among the participants, and they had been told about the screening *via* their families and coworkers. It was stated that 63% of participants reported feeling apprehensive while awaiting their screening results, but their anxiety levels subsided subsequently, and only a small percentage of carriers reported feeling less healthy. It was also notified that 97% of those tested reported no regrets regarding the test, and 97% would suggest it to

others. Additionally, the readers were informed that 94% of respondents agreed that couples should always seek pre-test counseling, and 83% stated they should seek it from a genetic counselor rather than their physician (*37*).

In a 2021 study by Bonneau *et al.* in France, it was shown that 91% of 1,568 participants had a favorable opinion of PCS, and 57% would be open to screening if it were available. A family doctor's or a gynecologist's medical prescription and social security insurance coverage for the test were reported to be the best ways to recommend testing, according to the majority of responders. Because of their ethical or moral convictions and concern that the results would cast doubt on the pregnancy, 19% of respondents claimed they were unwilling to be tested in the study. Despite the possibility that the results could medicalize the pregnancy, the majority of respondents viewed the test as a medical advancement. According to the findings, 65% of French physicians were not aware of this kind of test, and there was no discernible knowledge gap between them and the other respondents (*38*).

In a 2020 study conducted in the UK by Boardman *et al.*, the experiences and perspectives of a group of 20 thalassemia patients, those who have family members with thalassemia, and thalassemia carriers about preconception, prenatal, and neonatal thalassemia screening were examined. All prospective screening modalities were reported to receive a lot of encouragement because the majority of participants described thalassemia as a burdensome condition with a variety of adverse effects. However, particularly in religious communities, it was discovered that cultural, social, and, to a smaller degree, religious factors devalued the advantages of early screening, as stated by the study (*39*).

In 2021, Rabkina *et al.* surveyed 260 women who were nulliparous. 43.5% of respondents indicated that they were aware of carrier screening prior to the poll, and 77.8% indicated that they were interested in it. Reassurance and the desire to have information while making decisions about future pregnancies were listed as the key drivers of interest. A healthcare professional's in-person consultation was preferred (*40*).

Participants in a 2017 study by Chokoshvili *et al.* in Belgium were reported to show strong interest in reproductive genetic testing, such as prenatal testing and carrier screening for AR diseases/disorders, but low interest in genetic testing of newborns for susceptibility to adult-onset diseases/disorders. In addition, it was mentioned that there was a greater desire to undergo a predictive genetic test on oneself when the genetic testing is limited to ailments that are treatable or avoidable. According to the study, the vast majority of respondents stated that commercial offers of genetic testing through pharmacies or the internet were inappropriate and that genetic tests should instead be carried out in hospitals with a doctor's approval (*41*).

Carrier screening programs for rare diseases, like other diseases, are the result of a collaborative effort between multiple medical disciplines. In the existing literature, however, there are few studies that incorporate the views of many fields. There is also limited literature on carrier screening programs for rare diseases, as well as access to a representative sample of relevant government papers. It may be advantageous to identify the most effective carrier screening techniques for the needs of the countries, because the diseases expected to be seen may differ accordingly. These screenings should be in accordance with their national health policies and implemented as routine programs by conducting cost analyses along with the potential screening outcomes. All countries' genetic infrastructures should be thoroughly studied, and appropriate planning should be made by health professionals for diseases/disorders that can be detected through carrier screening, which can cause workforce loss, disease disability, and death, which may influence future generations in terms of public health, or whose disease outcomes might be disastrous and economically destructive and should be implemented as routine programs. Therefore, it is expected that carrier screening programs, similar to newborn blood spot screening, will become more widespread and that the examples of many countries will serve as a model for other countries in these efforts to protect public health (*29*).

10. Conclusion

In conclusion, this study has shed light on the disparities in carrier screening programs across countries, emphasizing the significant policy differences that exist. These variations, ranging from the involvement of health ministries to committee guidelines, university-level research, pilot studies, and individual scientists' research, underscore the low global priority and insufficient attention given to rare diseases.

Moving forward, further research should focus on establishing the necessary scientific infrastructure to develop and implement universal carrier screening programs. This effort is crucial for addressing inequalities in both local and global contexts, as health is a fundamental human right that should be ensured from birth. While common causes of mortality and morbidity such as chronic diseases and accidents receive considerable attention, rare diseases have a profound impact on individuals and society. They cause illness, disability, and death, leading to the loss of productivity, economic burdens, and psychological issues.

Rare diseases contribute significantly to the disease burden in terms of years lost due to premature death (YLL) and years lost due to disability (YLD). Neglecting rare diseases can result in severe healthcare challenges, especially in small-local communities. Therefore, it is imperative to define diagnosis, treatment, and

rehabilitation services for these diseases and address the social, psychological, and economic burden on caregivers in countries without carrier screening programs. For countries with existing programs, conducting a comparative cost analysis is necessary to allocate financial resources for individuals with the disease who were not screened and may have children.

Considering the high costs associated with diagnosing and treating rare diseases, it is crucial for international organizations to provide financial support to countries lacking sufficient economic infrastructure. By establishing and implementing carrier screening programs, the occurrence of diseases can be prevented, leading to improved health and overall societal well-being in future generations.

While this study serves as a guide for developing countries, it is essential to acknowledge that rare diseases pose a more severe challenge in underdeveloped regions where health problems often go undetected and health records are inadequate. However, rare diseases will not be noticeable until common mortality causes such as hunger, poverty, and acute infections are eliminated. Despite this study and other studies, rare diseases will continue to be the invisible face of the iceberg due to the nature of development differences between countries.

Funding: None.

Conflict of Interest: The authors have no conflicts of interest to disclose.

References

1. Julkowska D, Austin CP, Cutillo CM, Gancberg D, Hager C, Halftermeyer J, Jonker AH, Lau LPL, Norstedt I, Rath A, Schuster R, Simelyte E, van Weely S. The importance of international collaboration for rare diseases research: A European perspective. Gene Ther. 2017; 24:562-571.
2. Fidan Ç, Akdur R, Ünver ÇN, Şahin ÖC, Alper AB. The two essential components of a comprehensive rare disease control program. 6th International and 24th National Congress on Public Health. 2022. *https://uhsk.org/2022/davet/* (accessed August 16, 2023).
3. Taruscio D, Capozzoli F, Frank C. Rare diseases and orphan drugs. Ann Ist Super Sanità. 2011; 47:83-93.
4. de Vrueh R, Baekelandt ERF, de Haan JMH. Priority medicines for Europe and the world: a public health approach to innovation. WHO Background Paper 6.19. Rare Diseases. 2013.
5. U.S. Food and Drug Administration. Orphan Products: Hope for People With Rare Diseases. *https://www.fda.gov/drugs/information-consumers-and-patients-drugs/orphan-products-hope-people-rare-diseases* (accessed May 12, 2023).
6. Gregg AR, Aarabi M, Klugman S, Leach NT, Bashford MT, Goldwaser T, Chen E, Sparks TN, Reddi HV, Rajkovic A, Dungan JS; ACMG Professional Practice and Guidelines Committee. Screening for autosomal recessive and X-linked conditions during pregnancy and preconception: a practice resource of the American College of Medical Genetics and Genomics (ACMG). Genet Med. 2021; 23:1793-1806.
7. Molster CM, Lister K, Metternick-Jones S, Baynam G, Clarke AJ, Straub V, Dawkins HJS, Laing N. Outcomes of an international workshop on preconception expanded carrier screening: Some considerations for governments. Front Public Health. 2017; 5:25.
8. Rowe CA, Wright CF. Expanded universal carrier screening and its implementation within a publicly funded healthcare service. J Community Genet. 2020; 11:21-38.
9. Page MJ, McKenzie JE, Bossuyt PM, *et al.* The PRISMA 2020 statement: An updated guideline for reporting systematic reviews. BMJ. 2021; 372:n71.
10. Whiting P, Savovi J, Higgins JPT, Caldwell DM, Reeves BC, Shea B, Davies P, Kleijnen J, Churchill R. ROBIS: A new tool to assess risk of bias in systematic reviews was developed. J Clin Epidemiol. 2016; 69:225-234.
11. The American College of Obstetricians and Gynecologists. Carrier Screening for Genetic Conditions. *https://www.acog.org/clinical/clinical-guidance/committee-opinion/articles/2017/03/carrier-screening-for-genetic-conditions#s1* (accessed May 12, 2023).
12. Genetics Education Canada: Knowledge Organization. Point of care tool: Reproductive Genetic Carrier Screening in Canada. *https://geneticseducation.ca/resources-for-clinicians/prenatal-and-preconception-genomics/carrier-screening-in-canada/point-of-care-tool-14?highlight=WyJjYXJyaWVyIiwiJ2NhcnJpZXInIiwic2NyZWVuaW5nIiwiY2FycmllciBzY3JlZW5pbmciXQ==* (accessed August 16, 2023).
13. State of Israel Ministry of Health. Recommendations for Genetic Testing. *https://www.health.gov.il/English/Topics/Genetics/checks/Pages/GeneticTestingRecommendations.aspx* (accessed May 12, 2023).
14. Zlotogora J, Grotto I, Kaliner E, Gamzu R. The Israeli national population program of genetic carrier screening for reproductive purposes. Genet Med. 2016; 18:203-206.
15. Türkiye Cumhuriyeti Sağlık Bakanlığı. Hemoglobinopati Kontrol Programı. *https://hsgm.saglik.gov.tr/tr/cocukergen-tp-liste/hemoglobinopati-kontrol-program%C4%B1.html* (accessed May 12, 2023). (in Turkish)
16. Türkiye Cumhuriyeti Sağlık Bakanlığı. Evlilik Öncesi Spinal Musküler Atrofi (SMA) Taşıyıcı Tarama Programı. *https://hsgm.saglik.gov.tr/tr/cocukergen-tp-liste/evlilik-oncesi-spinal-muskuler-atrofi-sma-tasiyici-tarama-programi.html* (accessed May 12, 2023). (in Turkish)
17. Türkiye Cumhuriyeti Sağlık Bakanlığı. Evlilik Öncesi Hemoglobinopati Tarama Programı. *https://hsgm.saglik.gov.tr/tr/dokumanlar-cocukergen/egitim-dokumanlari.html* (accessed August 16, 2023). (in Turkish)
18. Istituto Superiore di Sanità. Italian External Quality Assessment of Genetic Testing. *https://www.iss.it/en/web/guest/archivio/-/asset_publisher/U2O0cK2cWrd9/content/il-ceq-istruzioni-1?_com_liferay_asset_publisher_web_portlet_AssetPublisherPortlet_INSTANCE_U2O0cK2cWrd9_assetEntryId=3447108&_com_liferay_asset_publisher_web_portlet_AssetPublisherPortlet_INSTANCE_U2O0cK2cWrd9_redirect=https%3A%2F%2Fwww.iss.it%2Fen%2Fweb%2Fguest%2Farchivio%3Fp_p_id%3Dcom_liferay_asset_publisher_web_portlet_AssetPublisherPortlet_INSTANCE_U2O0cK2cWrd9%26p_p_lifecycle%3D0%26p_p_state%3Dnormal%26p_p_mode%3Dview%26_com_liferay_asset_publisher_web_portlet_AssetPublisherPortlet_INSTANCE_U2O0cK2cWrd9_*

assetEntryId%3D3447108%26_com_liferay_asset_publisher_web_portlet_AssetPublisherPortlet_INSTANCE_U2O0cK2cWrd9_cur%3D0%26p_r_p_resetCur%3Dtrue (accessed August 16, 2023).
19. Ministerio de Salud de Italia. Piano per l'innovazione del sistema sanitario basato sulle scienze omiche. *https://www.salute.gov.it/portale/news/p3_2_1_1_1.jsp?id=3270&menu=notizie* (accessed August 16, 2023).
20. Amsterdam UMC Locatie AMC. De dragerschapstest. *https://www.amc.nl/web/dragerschapstesten/dragerschapstesten/de-dragerschapstest.htm* (accessed May 12, 2023). (in Dutch)
21. Victorian Clinical Genetics Services. Prepair carrier screening. *https://www.vcgs.org.au/prepair-carrier-screening/* (accessed August 16, 2023).
22. The Royal Australian College of General Practitioners. Genomics in general practice. *https://www.racgp.org.au/* (accessed August 16, 2023).
23. Royal Australian and New Zealand College of Obstetricians and Gynaecologists. Prenatal screening and diagnostic testing for fetal chromosomal and genetic conditions. *https://ranzcog.edu.au/* (accessed August 16, 2023).
24. Zhao S, Xiang J, Fan C, *et al.* Pilot study of expanded carrier screening for 11 recessive diseases in China: results from 10,476 ethnically diverse couples. Eur J Hum Genet. 2018; 27:254-262.
25. Netzer C, Velmans C, Erger F, Schreml J. Carrier testing for autosomal recessive disorders: A look at current practice in Germany. Medizinische Genetik. 2021; 33:13-19.
26. National Institutes of Health (NIH). Will health insurance cover the costs of genetic testing? *https://medlineplus.gov/genetics/understanding/testing/insurancecoverage/* (accessed May 12, 2023).
27. University Medical Center Groningen (UMCG). Dragerschapstest. *https://www.umcg.nl/-/dragerschapstest* (accessed May 12, 2023). (in Dutch)
28. United Kingdom National Health Service (NBS). Genetic and genomic testing. *https://www.nhs.uk/conditions/genetic-and-genomic-testing/* (accessed May 12, 2023).
29. Fidan Ç, Örün H, Alper AB, Ünver ÇN, Şahin ÖC, Uğurlu Z, Akdur R, Taruscio D. Expanded newborn bloodspot screening: developed country examples and what can be done in Turkey. Intractable Rare Dis Res. 2022; 11:63-69.
30. Türkiye Sağlık Enstitüleri Başkanlığı. Evlilik Öncesi Tarama Programı Tarihçesi, Mevzuatı, Mevcut Durumu ve Genişletme Modelleri. *https://files.tuseb.gov.tr/tacese/files/yayinlar/evlilik-oncesi-tarama-programi-tarihcesi-mevzuati-mevcut-durumu-ve-genisletme-modelleri.pdf* (accessed May 12, 2023).. (in Turkish)
31. Canatan D. Türkiye'de hemoglobinopatilerin epidemiyolojisi. HematoLog. 2014; 4-1:11-21. (in Turkish)
32. Verdonk P, Metselaar S, Storms O, Bartels E. Reproductive choices: A qualitative study of Dutch Moroccan and Turkish consanguineously married women's perspectives on preconception carrier screening. BMC Womens Health. 2018; 18:79.
33. Serim Timur. Türkiye'de Aile Yapısı. Ankara, Turkey, 1972. (in Turkish)
34. Türkiye İstatistik Kurumu (TÜİK) Kurumsal. İstatistiklerle Aile, 2020. *https://data.tuik.gov.tr/Bulten/Index?p=Istatistiklerle-Aile-2020-37251* (accessed May 12, 2023). (in Turkish)
35. Santos CM de A dos, Heller AH, Pena HB, Pena SDJ. A protocol for preconceptional screening of consanguineous couples using whole exome sequencing. Front Genet. 2021; 12:685123.
36. Cornel MC, Rigter T, Jansen ME, Henneman L. Neonatal and carrier screening for rare diseases: how innovation challenges screening criteria worldwide. J Community Genet. 2021; 12:257-265.
37. Mathijssen IB, Holtkamp KCA, Ottenheim CPE, Van Eeten-Nijman JMC, Lakeman P, Meijers-Heijboer H, van Maarle MC, Henneman L. Preconception carrier screening for multiple disorders: evaluation of a screening offer in a Dutch founder population. Eur J Hum Genet. 2018; 26:166-175.
38. Bonneau V, Nizon M, Latypova X, Gaultier A, Hoarau E, Bézieau S, Minguet G, Turrini M, Jourdain M, Isidor B. First French study relative to preconception genetic testing: 1500 general population participants' opinion. Orphanet J Rare Dis. 2021; 16:130.
39. Boardman FK, Clark C, Jungkurth E, Young PJ. Social and cultural influences on genetic screening programme acceptability: A mixed-methods study of the views of adults, carriers, and family members living with thalassemia in the UK. J Genet Couns. 2020; 29: 1026-1040.
40. Rabkina L, Swanson A, Aufox S, Propst L, Fiddler M, Wagner A, Arjunan A. What women want: General population perspectives and access to preconception expanded carrier screening. Prenat Diagn. 2021; 41:1057-1065.
41. Chokoshvili D, Belmans C, Poncelet R, Sanders S, Vaes D, Vears D, Janssens S, Huys I, Borry P. Public views on genetics and genetic testing: A survey of the general public in Belgium. Genet Test Mol Biomarkers. 2017; 21:195-201.

Received January 25, 2023; Revised August 18, 2023; Accepted August 20, 2023.

**Address correspondence to:*
Çağlar Fidan, Department of Public Health, Başkent University Faculty of Medicine, No: 38, Kapı No: 8-9, Bahçelievler, Ankara 06490, Turkey.
E-mail: caglarfidanmd@gmail.com

Released online in J-STAGE as advance publication August 22, 2023.

Review

DOI: 10.5582/irdr.2023.01020

VEXAS syndrome: Current clinical, diagnostic and treatment approaches

Senol Kobak*

Department of Internal Medicine and Rheumatology, Istinye University Faculty of Medicine, Liv Hospital, WASOG Sarcoidosis Clinic, Istanbul,Turkey.

SUMMARY VEXAS syndrome, is a hemato-inflammatory chronic disease characterized with predominantly rheumatic and hematologic systemic involvement. It was first described in 2020 by a group of researchers in the United States. VEXAS syndrome is a rare condition that primarily affects adult males and is caused by a mutation in the UBA1 gene located on the X chromosome. Its pathogenesis is related to the somatic mutation affecting methionine-41 (p.Met41) in UBA1, the major E1 enzyme that initiates ubiquitylation. Mutant gene lead to decreased ubiquitination and activated innate immune pathways and systemic inflammation occur. The specific mechanism by which the UBA1 mutation leads to the clinical features of VEXAS syndrome is not yet fully understood. VEXAS is a newly define adult-onset inflammatory syndrome manifested with treatment-refractory fevers, arthritis, chondritis, vasculitis, cytopenias, typical vacuoles in hematopetic precursor cells, neutrophilic cutaneous and pulmonary inflammation. Diagnosing VEXAS syndrome can be challenging due to its rarity and the overlap of symptoms with other inflammatory conditions. Genetic testing to identify the UBA1 gene mutation is essential for definitive diagnosis. Currently, there is no known cure for VEXAS syndrome, and treatment mainly focuses on managing the symptoms. This may involve the use of anti-inflammatory medications, immunosuppressive drugs, and supportive therapies tailored to the individual patient's needs. Due to the recent discovery of VEXAS syndrome, ongoing research is being conducted to better understand its pathogenesis, clinical features, and potential treatment options. In this review article, the clinical, diagnostic and treatment approaches of VEXAS syndrome were evaluated in the light of the latest literature data.

Keywords VEXAS syndrome, clinical, diagnositc, treatment, approaches

1. Introduction

In December 2020, a revolutionary study was shared with scientific community, which sheds light on the pathogenesis of some diseases and has strong evidence of gene-disease relationship (*1*). Using a genotype-driven approach, the authors identified a disorder that connects seemingly unrelated adult-onset inflammatory syndromes, named the VEXAS (vacuoles, E1 enzyme, X-linked, autoinflammatory, somatic) syndrome. The recurrent and acquired somatic mutations were found in UBA1, a gene encoding the ubiquitin-activating enzyme 1, which is necessary for the initiation of ubiquitylation (*2*). UBA1 is expressed as two isoforms (nuclear and cytoplasmic) differing in translation start site; nuclear UBA1a initiated at p.Met1 and cytoplasmic UBA1b initiated at p.Met41 (*3*). Ubiquitylation is a type of post-translational modification of proteins that is regulate the intracellular signaling and protein degradation through the proteasome or the autophagy-lysosome system (*4*). Loss of cytoplasmic UBA1 function and disruption of ubiquitylation process involving also myeloid lineage cells leading to uncontrolled inflammation resulting in a late-onset, treatment refractory inflammatory syndrome with associated hematologic abnormalities (*5*).

The possible role of various genes in the pathogenesis of rheumatological diseases has been investigated (*6*). As a result of genetic studies, monogenic (autoinflammatory) and/or polygenic (autoimmune) rheumatological diseases have been defined (*7*). Autoimmunity and autoinflammation were considered as "double-edged knife"; an one end of the "knife" are the various autoantibodies produced by B-cells and the other end are monogenic autoinflammatory diseases (*8*). Connective tissue diseases (ex. SLE) are the representative autoimmune diseases that depends on acquired immunity, whereas FMF are the representative of the autoinflammatory diseases in which the innate immunity

have important role (*9*). While the "borders" are so clearly have been drawn in rheumatology, some diseases have features belonging to both disease groups (*10*). With the definition of VEXAS syndrome, some "boundaries" in rheumatology knowledge have been crossed and the "walls" have been broken. This syndrome involving the clinical pictures of autoimmune and autoinflammatory diseases both opened new era in our knowledge.

Herein we review the genetic, immunological, clinical and treatment aspects of a newly defined VEXAS syndrome.

2. Focus on somatic mutations in rheumatology practice

It is well known that different genes play an important role in the pathogenesis of rheumatic diseases. Increased expression and/or gene polymorphisms of some genes have been shown to determine disease susceptibility, clinical course, and sometimes prognosis of the diseases (*11*). Rheumatological diseases can be polygenic and/or monogenic, and sometimes they can carry the characteristics of both groups (*12*). In general, the "borders are well drawn" in rheumatology and the diseases are well defined in terms of genetics. Autoimmune diseases are characterized by the synthesis of different B-lymphocyte-derived autoantibodies as a result of autoreactive T-cell activation triggered by an unknown antigen. It has been reported that many genes may be responsible for the pathogenesis of these diseases, not a single gene (*13*). It is well established that autoimmune diseases have common mechanisms and are caused by both genetic and non-genetic risk factors. One novel risk factor that can contribute to autoimmunity is somatic mutations, in a role parallel to their role in cancer (*14*). On the other hand, the development of autoinflammatory diseases has been described as a result of mutations in a single gene, the most striking example of which is FMF (*15*). Genetic mutations in the germline define an increasing list of heritable, monogenic autoinflammatory diseases that typically manifest early in life. In contrast to germline mutation, changes in DNA that occur after the first zygotic division are called somatic mutations (*16*).

Unlike germinal mutations, somatic mutations are genomic alterations that are not transmitted to offspring. Its may occur during life, from early embryogenesis through adulthood (*17*). Somatic mutations are usually restricted to specific tissue types, and may play a causal and important role in non-heritable rheumatological diseases, especially conditions that start in advanced stage of life (*18*). Using sequencing technology it is possible to detect somatic mutations in various tissue types, especially blood (*19*). While somatic mutations are well defined in malignant hematological diseases, its role in rheumatological diseases is not clearly yet (*20*). Although rare, some autoimune and autoinflammatory rheumatic diseases associated with somatic mutation have been described (Table 1).

Schnitzler syndrome is a rare adult-onset autoinflammatory disease characterized with chronic urticarial rash, recurrent fever, arthralgia or arthritis, monoclonal gammopathy of undetermined significance (MGUS), and marked systemic inflammation (*21*). The

Table 1. Somatic mutations in rheumatic diseases

Disease	Gene	Chromosome	Mechanism	Clinical findings	Treatment
VEXAS syndrome	UBA1	Chr. X	LOF	recurrent fever, polychondritis, vasculitis, arthritis, macrocytic anemia	CSs Tocilizumab JAKi Azacytidine ASCT
MDS/BD	NR	Crh.8	trisomy	oral/genital ulcers, rash, fever and intestinal involvement	DMARDs chemoterapeutic drugs
ECD	MAPK/BRAF	Crh.7	GOF	central diabetes insipidus, restrictive pericarditis, perinephric fibrosis, and sclerotic bone lesions	BRAF inhibitors
TRAPS	TNFRSF1A	Chr.12	GOF	recurrent fever; abdominal, chest, and muscle pain; red and swollen eyes; and a typical rash lasting for more than one week.	IL-1 blockers (ANK, CKM) TNFA inh.(ETN) IL-6 inh (TCZ)
NLRP3-AID	NLRP3	Chr. 1	GOF	fever, urticaria,arthritis sensorineural hearing loss, central nervous system involvement	IL-1 blockers (ANK, CKM)
ALPS	FAS	Chr. 10	LOF	lymphadenopathy and splenomegaly, hypergammaglobulinemia, haemolytic anaemia, idiopathic thrombocytopenia and neutropenia	CSs IS drugs splenectomy
Felty s/m	STAT 3	Chr .7	GOF	splenomegaly, anemia, neutropenia, thrombositopenia	DMARDs, splenectomy IL-1 blockers
Schnitzler syndrome	MYD88	Chr.1	GOF	chronic urticarial rash, recurrent fever, arthralgia or arthritis, monoclonal gammopathy of undetermined significance (MGUS)	(ANK, CKM)

AID: NLRP3-associated inflammatory disease; ALPS: autoimmune lymphoproliferative syndrome; ANK:anakinra; ASCT: allogeneic hematopoietic stem cell transplantation; CKM:canakinumab; CSs:corticosteroids; DMARDs:disease modifying anti-rheumatic drugs; ECD: Erdheim-Chester disease; ETN:etanercept; GOF: gain-of-function; IL-1:interleukine-1; JAKi: janus kinase inhibitors; LOF: loss-of-function; MAPK: mitogen activated protein kinase; MDS: myelodysplastic syndrome; NLRP3- TRAPS: tumour necrosis factor receptor-associated periodic syndrome; TCZ: tocilizumab; TNFA: tumor necrosis factor alpha; VEXAS: vacuoles, E1enzyme, X-linked, autoinflammatory, somatic.

inflammasome related IL-1 overproduction is a result of a somatic mosaic gain of function mutation of NLRP3 (nucleotide-binding oligomerization domain [NOD]-like receptor [NLR] family pyrin domain containing 3) gene. Somatic NLRP3 mosaicism was found also in patients with Schnitzler-like syndromes. Patients with IgGκ variant Schnitzler syndrome and severe clinical phenotype showed myeloid lineage restrict somatic NLRP3 mosaicism. Mutated NLRP3 gene (c.1906C >G p.Q636E) resulted in cell death in a monocyte cell line, and adaptor molecule apoptosis-associated speck-like protein containing a CARD (ASC)-dependent activation of nuclear factor (NF)-κB (*22*). This resulted in inflammasome activation and overproduction of predominantly IL-1 cytokine. There are report that some patients with Schnitzler syndrome have MYD88 gene somatic mutations, which is considered an independent risk factor for Waldenström's macroglobulinemia (WM) (*23*). That data may be useful to guide clinical monitoring since a significant proportion of patients with Schnitzler syndrome might develop lymphoproliferative malignancy.

The autoimmune lymphoproliferative syndrome (ALPS), which is the first identified non-malignant autoimmune disease caused by a somatic mutation, is characterized with persistent lymphadenopathy and splenomegaly, hypergammaglobulinemia, haemolytic anaemia, idiopathic thrombocytopenia and neutropenia (*24*). The cause of ALPS was found to be somatic FAS mutations in hematopoietic precursor stem cells, an accumulation of double-negative T cells, and hypergammaglobulinemia. Savola *et al.* reported somatic mutations in immune- related genes in mature expanded CD8+ T-cell populations in RA patients (*25*). They conclude that this mutations were associated with autoimmunity or cell survival. The same authors showed the somatic STAT3 mutations in Felty's syndrome shares molecular markers with large granular lymphocyte (LGL). The authors conclude that this findings provided molecular evidence for the hypothesis that Felty's syndrome and LGL leukemia are actually the same disease (*26*). Monogenic autoinflammatory diseases are group of familial and sporadic autosomal dominant diseases characterized by gain-of-function mutations in NLRP3, leading to increased inflammasome activity (*27*). Some NLRP3 mutations are closely related to disease severity, while other are associated with heterogeneous clinical presentations, due to various genetic or environmental factors. Sporadic NLRP3-AID may be due to de novo germline or acquired somatic mutations (*28*). While NLRP3 somatic mutations have mostly been described in childhood patients these mutations may also cause adult-onset disease. Using conventional Sanger sequencing, a large international study investigators identified that 30% of neonatal-onset multisystem inflammatory disorder (NOMID) cases are due to mosaicism (*29*). Sporadic NOMID clinical features is shown to be more aggressive and severe than familial form.

Erdheim-Chester disease (ECD) is a rare histiocytosis characterized with systemic inflammatory features involving skin, lung, aorta, bone, central nervous system and the retroperitoneum (*30*). Typical findings of ECD include central diabetes insipidus, restrictive pericarditis, perinephric fibrosis, and sclerotic bone lesions. Somatic mutations activating the MAPK pathway are found in more than 80% of patients with ECD, mainly the BRAF activating mutation, followed by MAP2K1 (*31*). Multisystem or refractory ECD patents have benefitted from highly effective therapy with BRAF and MEK inhibitors.

Recently the trisomy 8 mosaicism (T8m) was reported in patients with Behçet disease (BD). T8m has a highly variable phenotype, and it may also be associated with haematological disorders, such as MDS. It was reported that Behçet-T8m patients have mostly oral/genital ulcers, rash, fever and intestinal involvement (*32*). In recent years our knowledges about somatic mutations associated with various rheumatological diseases are increased. Somatic mutations seen in autoimmune and autoinflammatory diseases may also act as a "bridge" in terms of inflammation and malignancy development. The newly described VEXAS syndrome is not only a disease that culminates this relationship, but also a new beginning in this regard.

3. Genetic and immunological aspect of VEXAS syndrome

VEXAS syndrome is adult onset hematoinflammatory disease caused by somatic mutations in the gene UBA1, the majör E1 enzyme that initiates ubiquitylation. Ubiquitylation is a post-translational modification that triggers proteasomal degradation (*33*). It is essential for various cellular processes such as cell cycle progression, DNA damage response, and inflammatory signaling pathways (*34*). Dysregulation of the ubiquitin-proteasome system results in susceptibility to infection, lymphoproliferative disorders, autoinflammatory diseases and malignancy (*35*). Ubiquitylation is initiated by the attachment of a single ubiquitin molecule to a target protein through a three-step process performed by the concerted actions of ubiquitin activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and substrate specific ligases (E3). Ubiquitin-like modifier-activating enzyme 1 (UBA1), the major E1 enzyme, has two isoforms: UBA1a and UBA1b (*36*). UBA1a is the long isoform and is localized in the nucleus, whereas UBA1b is the short isoform and is localized in the cytoplasm without a nuclear localization signal.

The major cause of the VEXAS syndrome is a depletion of cytoplasmic UBA1 which resultant decreased ubiquitylation activates the unfolded protein response and type I interferon production (*37*). Increase

inflammation in the VEXAS syndrome is driven by mutant myeloid cells which can survive with this somatic mutation. Patients with VEXAS syndrome have highly activated inflammatory pathways including tumor necrosis factor, interleukin-6, and interferon-γ, which a finding that is consistent with severe myeloid inflammation (*38*). Activation of multiple cytokines cascades result in the elevated acute phase reactants (C-reactive protein and erythrocyte sedimentation rate) levels in the sera which is the characteristic laboratory findings in VEXAS patients (*39*). The changes were also seen in B-lymphocytes repertoar, atypical differentiation of B-cells with loss of immature B cells and increase of monocyte populations (*40*). This increase inflammatory responses in VEXAS syndrome may also aggravated by activation of neutrophils with preserved phagocytic activity and formation of neutrophil extracellular traps (NETs) (*41*).

4. Clinical features of VEXAS

VEXAS syndrome is an adult-onset inflammatory syndromes often manifest with overlapping rheumatologic and hematologic clinical features. Typically, this condition predominantly affects middle-aged and older men, occurring in the fifth and seventh decade of their lives. The exact prevalence of the syndrome in the population is still unknown. Initially, it was thought to be exclusive to males due to the involvement of the X chromosome (UBA1 gene). However, female cases with inherited or acquired monosomy of the X chromosome have also been reported (*42,43*).

VEXAS is a heterogeneous syndrome that can manifest with various hematological and rheumatic manifestations (*44*). The hematological features often include progressive abnormalities like macrocytic anemia, thrombocytopenia, myeloid dysplasia, and bone marrow vacuolization affecting myeloid and erythroid precursor cells (*45*). The most common clinical features comprise recurrent fever, arthralgia/arthritis, pulmonary involvement, skin lesions, various types of vasculitis, and/or thromboembolic events (*46*). From a rheumatologist's perspective, VEXAS syndrome can mimic known rheumatologic diseases or coexist with them (*47,48*) (Table 2). Since the recognition of this syndrome, many rheumatologists have reviewed their previous diagnoses. VEXAS can present with clinical findings seen in various rheumatological diseases, leading to misdiagnoses before its definition. Notably, patients previously diagnosed with vasculitis, connective tissue disease, and/or autoinflammatory disease were later identified to have VEXAS syndrome based on recent data (*49*). Interestingly, VEXAS patients may also partially meet established diagnostic or classification criteria for several known clinical conditions (*50*).

Given its heterogeneity, VEXAS syndrome exhibits a wide range of clinical findings. Numerous rheumatology centers have reported their cases since the disease's recognition (Table 3). In the first original VEXAS study by Beck *et al*. among 25 patients 18 (72%) had pulmonary involvement, 16 (64%) had ear and nose chondritis, four patients had vasculitis (3PAN, 1GCA) (*1*). Subgroups of participants met or partially met established diagnostic or classification criteria for a number of clinical conditions (ex. PAN, GCA, relapsing polychondritis). Coster *et al.* reported the clinical features and outcomes of 9 patients with VEXAS syndrome followed at Mayo Hospital (*51*). Vasculitis was observed in 4 patients (cutaneous, renal peritubular capillaritis, cryoglobulinemia and large vessel vasculitis involving abdominal aorta). Reccurent ear and nose chondritis was present in 5 of 9 patients; ocular inflammation was observed in 4 patients (2 with uveitis and 2 with episcleritis). All patients with ear and nose chondritis, ocular inflammation, and vasculitis had the p.Met41Thrn mutation. Ciferska *et al.* reported 3patients newly diagnosed as VEXAS syndrome in their rheumatology center (*52*). All 3 patients are elderly man, the median age of the patients are 72.6 years old. The patients have been diagnosed with a different rheumatic disease (1 seronegative RA, 1 relapsing polychondritis, 1 paraneoplastic s/m) before VEXAS diagnosis. All 3 patients expressed multiple inflammatory manifestations exceeding the typical clinical phenotypes associated with the referred rheumatic diagnoses, and all had additional haematological abnormalities. Khitri *et al.* compared the clinical characteristics, the laboratory features and the outcomes between idiopathic-relapsing polychondritis (I-RP) and VEXAS-relapsing polychondritis (VEXAS-RP) (*53*). They found that VEXAS-RP characterized by high prevalence of male sex, fever, skin lesion, ocular involvement, pulmonary infiltration, heart involvement, older age and hematological abnormality. Staels *et al.* reported two VEXAS patients; one characterized by recurrent rash and symmetric polyarthritis, and another who was initially diagnosed with idiopathic multicentric Castleman disease and developed macrophage activation syndrome as a complication of the VEXAS syndrome

Table 2. Rheumatic manifestations of VEXAS syndrome

Clinical Manifestations	Associated Conditions
Recurrent fever	Coexistance with:
Relapsing polychondritis	- Systemic lupus erythematosus
Polyarteritis nodosa	- Still disease
Giant cell arteritis	- Vasculitides
Arthralgia	- Relapsing polychondritis
Inflammatory asymetric mono/oligoarthritis	- Macrophage activation syndrome
Neutrophilic alveolitis	- Spondylarthritis
Venous thrombosis	- Hematological malignancy
Lymphadenopathy	
Scleritis/episcleritis/uveitis/retinal vasculitis	
Sweet syndrome	
AA amyloidosis	

Table 3. Published studies in the literature showed the main characteristics of patients with VEXAS syndrome

Features	Beck *et al.* *Ref (1)*	van der Made *et al.* *Ref (56)*	Muratore *et al.* *Ref (57)*	Khitri *et al.* *Ref (53)*	Ferrada *et al.* *Ref (60)*	Tsuchida *et al.* *Ref (59)*	Bourbon *et al.* *Ref (58)*	Ciferska *et al.* *Ref (52)*	Koster *et al.* *Ref (51)*
Gender (M/F), *n* (%)	25 (100%)	12 (100%)	7 (100%)	53 (96%)	13 (100%)	8 (100%)	11 (100%)	3 (100%)	9 (100%)
Median age at onset, years	64 (45–80)	67 (55–79)	64 (46–76)	66 (61–72)	62 (48–71)	72 (66–81)	66 (47–83)	74 (68–76)	70 (65–72.5)
Fever, *n* (%)	23 (92%)	11 (92%)	7 (100%)	33 (60%)	13 (100%)	6 (75%)	10 (91%)	1 (33%)	8 (88%)
Artralgia/Arthritis, *n* (%)	NR	4 (33%)	NR	36 (67%)	6 (46%)	2 (25%)	11 (100%)	1 (33%)	5 (55%)
Vasculitis, *n* (%)	4 (16%)	2 (16%)	7 (100%)	3 (6%)	NR	NR	7 (64%)	3 (100%)	4 (44%)
Eyes involvement, *n* (%)	NR	5 (42%)	NR	30 (57%)	NR	3 (38%)	5 (46%)	2 (67%)	4 (44%)
Skin involvement, *n* (%)	22 (88%)	10 (83%)	5 (71%)	44 (82%)	11 (85%)	7 (88%)	11 (100%)	3 (100%)	NR
Pulmonary, *n* (%)	18 (72%)	8 (67%)	6 (85%)	13 (46%)	10 (77%)	NR	5 (46%)	1 (33%)	NR
Chondritis, *n* (%)	16 (64%)	6 (50%)	1 (14%)	52 (98%)	13 (100%)	8 (100%)	5 (46%)	3 (100%)	5 (55%)
Hematologic, *n* (%)	macrocytic anaemia 24 (96%), MDS 6 (24%), MM or MGUS 5 (20%)	Macrocytic anemia 11 (%) MDS 6 (%)	Macrocytic anemia 6 (%)	MDS 41 (75%)	MDS 3 (23%) MGUS 1 (8%) MM 1 (8%)	macrocytic anaemia 7 (88%), MDS 4 (50%)	macrocytic anaemia 7 (64%), MDS 6 (54%)	macrocytic anaemia 2 (67%) MDS 1 (33%)	macrocytic anemia 9 (100%) MM 1 (%) MDS 1 (%)
UBA mutation, *n* (%)	p.Met41Thr 15 (60%) p.Met41Val 5 (20%) p.Met41Leu 5 (20%)	p.(Met41Thr), 12 (%)	p.Met41Thr	NR	p.Met41Thr 8 (62%) p.Met41Val 2 (15%) p.Met41Leu 3 (23%)	p.Met41Thr 3 (37.5%) p.Met41Val 2 (25%) p.Met41Leu 3 (37.5%)	p.Met41Thr 5 (46%) p.Met41Val 3 (27%) p.Met41Leu 1 (9%)	p.Met41Thr 2 (67%) p.Met41Leu 1 (33%)	p.Met41Thr 9 (100%)
CRP (median) (mg/L)	73 (18–128)	185 (61–407)	152 (41–250)	69 (30–107)	17.7 (9.6–99.5)	NR	114 (16.7-205)	73 (63-89)	NR
ESR (median) mm/h	97 (64–124)	111 (71–130)	94 (50–137)	NR	66.5 (42–110)	NR	NR	NR	NR

CRP:C-reactive protein; ESR: erythrocyte sedimentation rate; MDS: myelodysplastic syndrome; MGUS: monoclonal gammopathy of undetermined significance: UBA: ubiquitin-associated domain; NR:not reported.

(*54*). The latter patients was treated with anti-IL6 therapy (siltuximab) leading to a regression of systemic symptoms. Oganesyan *et al.* described the 76 years old male patients with recurrent fevers, joint inflammation, elevated levels of acute-phase reactants, macrocytic anaemia and skin lesions, who was diagnosed with VEXAS syndrome (*55*). The patient have been treated with anakinra (anti-IL1R antagonist) first, but did not result in any clinical or biological response. Prednisolone (1 mg/kg per day) was started and rapidly both clinical and biological complete remission within the week was done. Van der Made *et al*. reported 12 patients with VEXAS syndrome who had previously been registered as having unclassified autoinflammation (*56*). All patients were male and the median age were 67 years old (range 47–79 years). The patients presented with systemic symptoms, elevated inflammatory parameters, and multiorgan involvement. The newly reported features of VEXAS from this study included interstitial nephritis, cardiac involvement, stroke, and intestinal perforation related to treatment with tocilizumab. Despite different types of treatment were initiated, most patients became treatment-refractory, with a high mortality rate of 50%. Sharma *et al.* reported the coexistence of VEXAS syndrome and SLE in 70 years-old male (*47*). He was initiated on monthly intravenous immunoglobulin and clinical and laboratory regression was achieved. Recently Muratore *et al*. retrospectively evaluated the clinical records of 147 consecutive male patients followed up in their vasculitis clinic from 2013–2022. The authors identified seven patients (one with AAV) with vasculitis and concomitant features of VEXAS syndrome (*57*). Bourbon *et al.* identified 19 male patients with myeloid dysplasia and autoinflammatory disease such as relapsing polychondritis or Sweet syndrome. Among these 19 patients, 11 (57.9%) had a mutation of UBA1 gene (*58*). The median age at disease onset was 66 years (range, 47–83 years). As expected, most patients had fever (91%), skin involvement (100%), and arthritis or arthralgia (100%). All patients had an elevated serum concentrations of C-reactive protein, and most of them (64%) had macrocytic anemia. Tsuchida *et al.* reported the association of VEXAS syndrome and relapsing polychondritis (RP) in Japanese patients (*59*). UBA1 was examined in 13 of the 14 patients; 73% (8/11) of the male patients had somatic UBA1 variants. The authors concluded that genetic screening for pathogenic UBA1 variants should be considered in patients with RP, especially male patients with skin lesions. Ferrada *et al.* reported the prevalence of somatic mutations in UBA1 in patients cohort with RP (*60*). Seven of 92 patients with RP (7.6%) had UBA1 mutations (VEXAS-RP). Patients with VEXAS-RP were all male, were on average ≥ 45 years of age at disease onset, and commonly had fever, ear chondritis, skin involvement, deep vein thrombosis, and pulmonary infiltrates. Mortality was greater in VEXAS-RP than in RP (23% *vs.* 4%).

Table 4. Proposed algorithm to identify patients with VEXAS syndrome

Demographic	Clinical	Coexistence with	Laboratory Findings	Genetic Findings
Age: older age group	Unexplained recurrent fever	Relapsing polychondritis resistant to treatment and/or with a different clinical course	Increase serum cytokines (IL-1, IL-6, IL-17, TNF-alpha)	UBA1 mutation
Gender: predominantly male	Recurrent polychondritis (ear-nose)	Treatment-resistant vasculitis, which is beyond the classical knowledge	Unexplained very high CRP/ESR	
Population: Caucasian	Inflammatory arthritis: usually involving large joints of the lower extremities	Spondylarthritis or connective tissue diseases (ex. SLE)	Macrocytic anemia	
	Vasculitis: in the presence of simultaneous involvement of vessels of different diameters	Hematological malignancy (MDS) with signs of autoimmune disease	Presence of typical vacuoles in bone marrow biopsy	
	Eye involvement: treatment-resistant scleritis/episcleritis/retinal vasculitis			
	Severe, treatment-resistant skin lesions(inc. leucocytoclastic vasculitis and Sweet s/m)			
	Lung involvement, neutrophilic alveolitis			
	Kidney involvement, interstitial nephritis			

CRP:C-reactive protein; ESR:erythrocyte sedimentation rate; IL-1:interleukine-1; IL-6:interleukine-6; IL-17:interleukine-17; MDS: myelodisplastic syndrome; SLE:systemic lupus erythematosus;TNF-alpha:tumor necrosis factor-alpha; UBA1: Ubiquitin-like modifier-activating enzyme 1.

Georgin-Lavialle *et al.* reported the characteristics of one hundred and sixteen patients with VEXAS syndrome from a French multicentre registry between November 2020 and May 2021 (*61*). The main clinical features of VEXAS syndrome were found to be skin lesions (83%), noninfectious fever (64%), weight loss (62%), lung involvement (50%), ocular symptoms (39%), relapsing chondritis (36%), venous thrombosis (35%), lymph nodes (34%) and arthralgia (27%). The studies report that VEXAS syndrome may mimicking and/or coexist with any type of vasculitis (*62,63*). Watanabe *et al.* reviewed the literature data to delineate the clinical characteristics of vasculitis associated with VEXAS syndrome (*64*). The authors reported 23 patients with VEXAS-vasculitis. Among 23 patients, 2 (9%) had LVV, 9 (39%) had medium vessel vasculitis, and 12 (52%) had small vessel vasculitis. The median age of the patients were 66.4 years old (range 50–87). The AutoInflammatory Disease Alliance (AIDA) international Registry for VEXAS syndrome included 113 Centers from 23 Countries in 4 continents, is designed for the retrospective and prospective collection of real-life data (*65*). The aim of this registry is to collect the demographic, genetic, clinical, laboratory and treatment data starting since the disease. The rheumatology community awaits the results of this registry with impatience, curiosity and excitement.

In conclusion, rheumatologists should consider the possibility of VEXAS syndrome in the following situations: *i*) elderly men, particularly those in their fifth decade of life or older; *ii*) patients presenting with clinical features of adult-onset autoinflammation accompanied by multiorgan involvement including recurrent fever, skin inflammation, asymmetrical mono/oligoarthritis, relapsing polychondritis with pulmonary involvement, any type of vasculitis, and MDS-like features observed in peripheral blood (Table 4).

Moreover, it is essential to be aware that VEXAS patients often prove to be treatment-refractory, despite attempts with multiple drugs. Given the overlapping clinical features with other rheumatologic diseases, keeping VEXAS syndrome in mind will facilitate accurate diagnosis and appropriate management for these patients. Early recognition of VEXAS can help improve patient outcomes and quality of life by tailoring treatment strategies to address the unique challenges posed by this heterogeneous syndrome.

5. VEXAS syndrome: more questions than answers

VEXAS syndrome is a recently defined disease with several uncertainties and unknown aspects, making each new case report invaluable and significant in shaping future research. As more cases are reported, we gain insights into the heterogeneous and complex nature of VEXAS syndrome (*66*). Despite its seemingly benign appearance, VEXAS is a condition associated with high morbidity and mortality due to its involvement across multiple body systems (*67*). It serves as a "bridge" between autoimmunity, autoinflammation, and carcinogenesis, with the prognosis heavily influenced by hematological involvement and/or the development of malignancy. From a rheumatological standpoint, it is essential to re-evaluate older age patients for the possibility of VEXAS, even if they were previously diagnosed with vasculitis, autoinflammatory diseases, or recurrent polychondritis according to the ACR/EULAR classification criteria (*68*). Another unresolved area pertains to identifying risk factors that influence

the disease's course, prognosis, and the development of malignancy in VEXAS syndrome. It can manifest with mild symptoms or life-threatening clinical presentations. To pave the way for future randomized prospective studies on VEXAS, it is crucial to establish clear and defined diagnosis/classification criteria for the disease. This will enable the recognition of distinct disease phenotypes and, more importantly, the determination of treatment options and protocols. The advancement of new genetic methods, including next-generation sequencing, will not only aid in diagnosing VEXAS but also uncover numerous other rheumatological diseases associated with somatic mutations. This promising avenue of research holds the potential to revolutionize our understanding and management of various rheumatologic conditions.

6. Treatment

Currently, there are no established treatment protocols for this newly defined disease in the medical literature. As randomized controlled trials have not yet been conducted, the treatment of VEXAS syndrome is primarily based on the collective clinical experience gained from treating autoinflammatory diseases and the information available from recently reported VEXAS case series (*69,70*).

As mentioned earlier, VEXAS syndrome has shown resistance to multiple therapeutic agents, resulting in high mortality rates. The disease's complexity, involving multiple mechanisms, may contribute to its relative resistance to various target-specific anti-rheumatic agents, with the exception of systemic glucocorticoids (*71*). The one approach that has been considered based on the increased serum IL-6 levels and significantly elevated CRP levels observed in VEXAS patients is the use of tocilizumab. Tocilizumab may be considered as a drug of choice for some manifestations of the syndrome; however, it does not appear to alter the disease's progression (*72*). There are various report of tocilizumab use in patients with VEXAS syndrome. Goyal *et al.* reported the efficacy of tocilizumab for treatment of cutaneous and systemic manifestations of VEXAS syndrome (*73*). Despite treatment with methotrexate and mycophenolate mofetil, the patient remained dependent on high doses of systemic corticosteroids. Weekly injections of tocilizumab were initiated and daily oral prednisone was continued. At 6.month of tocilizumab treatment the patient complaints disappear almost totally. Kunishita *et al.* reported the efficacy and safety of tocilizumab (TCZ) and CSs in 3patients with VEXAS syndrome (*74*). One-years follow-up showed that the combination of TCZ and glucocorticoids allowed the patients to continue treatment for at least one year without significant disease progression. Glucocorticoids were able to be reduced from the start of TCZ. Indeed, Janus kinase inhibitors (JAKi) have shown promise as potential treatment options for VEXAS syndrome. JAK inhibitors work by blocking intracellular signaling pathways activated by cytokine receptors, leading to decreased gene activation and subsequent immune responses. Several studies and case reports have suggested that JAK inhibitors could be clinically effective in managing VEXAS (*75-78*). Heiblig *et al.* reported the efficacy of JAKi in 30 patients with VEXAS syndrome (*79*). The authors describe more significant treatment efficacy with the JAK1/2 inhibitor ruxolitinib compare with other JAKi. Rates of clinical remission favored ruxolitinib over other JAK inhibitors at 67% *vs.* 38% at month 1, 83% *vs.* 18% at month 3, and 87% *vs.* 11% at month 6. There was also a marked steroid dose reduction of 83.6% with ruxolitinib and 75% with other JAK inhibitors.

Indeed, in some patients with VEXAS syndrome, benefits from intravenous immunoglobulin (IVIG) administration have been reported. IVIG is a treatment approach that involves infusing a solution containing pooled immunoglobulins obtained from the plasma of healthy donors. It is known for its immunomodulatory and anti-inflammatory effects. Magnol *et al.* reported a case previously diagnosed with spondyloarthritis and VEXAS syndrome (*80*). Despite treatment with cDMARDS and bDMARDs(anti-TNF-inhibitor) and tDMARDS (baricitinib), the patients symptoms have been continued. Intravenous immunoglobuline and IL-17 inhibitor have been started and the patients complaints disappear. The treatment of VEXAS syndrome can be particularly challenging due to the varying clinical presentations and the limited response of certain manifestations to targeted therapies like biologic disease-modifying antirheumatic drugs (b/tDMARDs). While b/tDMARDs may show some efficacy in managing milder skin or rheumatic manifestations of the disease, they may not be as effective in addressing the hematological features associated with VEXAS. One such approach that has shown promise in some cases is the use of azacytidine, a hypomethylating agent. Azacytidine is commonly used in the treatment of myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML) and has demonstrated potential in managing VEXAS-related hematological abnormalities (*81-83*). Based on a French nationwide registry of 116 patients with VEXAS, Comont *et al.* report the efficacy and safety of azacitidine in 11 patients with VEXAS concomitant with MDS (*84*). Clinical response of VEXAS to azacitidine was achieved in five patients (46%), suggesting that azacitidine can be effective in selected patients with VEXAS and associated MDS. For severe cases of VEXAS syndrome with widespread multi-systemic involvement, including severe hematological abnormalities, bone marrow transplantation may be considered as a curative therapeutic option (*85*). Diarra *et al.* reported successful allogeneic hematopoietic stem cell transplantation(ASCT) in 4patients with VEXAS syndrome (*86*). Three patients are in durable complete remission after ASCT. One unfortunately

died post-ASCT. The authors suggest that ASCT could be a curative option in patients with VEXAS syndrome and severe manifestations. Given the lack of established treatment guidelines, managing VEXAS syndrome remains a significant challenge for healthcare professionals. Continued research, case reports, and clinical experience will be critical in shaping the development of effective treatment strategies to improve the prognosis and quality of life for individuals affected by this complex and potentially life-threatening condition.

7. Conclusions

It is clear that somatic mutations in rheumatic diseases are more likely to have more severe functional effects than germinal mutations, since they do not undergo purifying selection of the whole organism.

VEXAS syndrome, which is caused by myeloid-restricted somatic missense mutations in UBA1, may cause severe inflammatory conditions that manifest in adulthood. The presence of recurrent fever, relapsing polychondritis, arthritis, vasculitis, macrocytic anemia, elderly male, and symptoms refractory to traditional steroid-sparing agents should prompt rheumatologists to consider VEXAS as a possible diagnosis. The treatment of VEXAS syndrome is not known yet, the beneficial role of corticosteroids, b/tDMARDs (tocilizumab, JAKi)), various chemotherapeutics (azacytidine) and bone marrow transplantion has been reported. More genetic studies and close collaboration between hematologists and rheumatologists are needed to identify and characterize the clinical phenotypes and treatment approaches of VEXAS syndrome.

Funding: None.

Conflict of Interest: The authors have no conflicts of interest to disclose.

References

1. Beck DB, Ferrada MA, Sikora KA, *et al.* Somatic mutations in *UBA1* and severe adult-onset autoinflammatory disease. N Engl J Med. 2020; 383:2628-2638.
2. Poulter JA, Collins JC, Cargo C, De Tute RM, Evans P, Ospina Cardona D, Bowen DT, Cunnington JR, Baguley E, Quinn M, Green M, McGonagle D, Beck DB, Werner A, Savic S. Novel somatic mutations in UBA1 as a cause of VEXAS syndrome. Blood. 2021; 137:3676-3681.
3. Komander D, Rape M. The ubiquitin code. Annu Rev Biochem. 2012; 81:203-229.
4. Beck DB, Werner A, Kastner DL, Aksentijevich I. Disorders of ubiquitylation: Unchained inflammation. Nat Rev Rheumatol. 2022; 18:435-447.
5. Craney A, Rape M. Dynamic regulation of ubiquitin-dependent cell cycle control. Curr Opin Cell Biol. 2013; 25:704-710.
6. Gerussi A, Soskic B, Asselta R, Invernizzi P, Gershwin ME. GWAS and autoimmunity: What have we learned and what next. J Autoimmun. 2022; 133:102922.
7. Gaggiano C, Vitale A, Tufan A, *et al.* The autoinflammatory diseases alliance registry of monogenic autoinflammatory diseases. Front Med (Lausanne). 2022; 9:980679.
8. Hocking AM, Buckner JH. Genetic basis of defects in immune tolerance underlying the development of autoimmunity. Front Immunol. 2022; 13:972121.
9. Efthimiou P, Petryna O, Nakasato P, Kontzias A. New insights on multigenic autoinflammatory diseases. Ther Adv Musculoskelet Dis. 2022; 14:1759720X221117880.
10. MacLauchlan S, Fitzgerald KA, Gravallese EM. intracellular sensing of DNA in autoinflammation and autoimmunity. Arthritis Rheumatol. 2022; 74:1615-1624.
11. Mikhaylenko DS, Nemtsova MV, Bure IV, *et al.* Genetic polymorphisms associated with rheumatoidarthritis development and antirheumatic therapy response. Int J Mol Sci. 2020; 21:4911.
12. Ishigaki K. Beyond GWAS: From simple associations to functional insights. Semin Immunopathol. 2022; 44:3-14.
13. Reed JH. Transforming mutations in the development of pathogenic B cell clones and autoantibodies. Immunol Rev. 2022; 307:101-115.
14. Rosen A. Immunoediting in autoimmune rheumatic diseases. Trans Am Clin Climatol Assoc. 2022; 132:34-43.
15. Ozen S. Update in Familial Mediterranean fever. Curr Opin Rheumatol. 2021; 33:398-402.
16. Ren P, Dong X, Vijg J. Age-related somatic mutation burden in human tissues. Front Aging. 2022; 3:1018119.
17. Evans MA, Walsh K. Clonal hematopoiesis, somatic mosaicism, and age-associated disease. Physiol Rev. 2023; 103:649-716.
18. Abbasi A, Alexandrov LB. Significance and limitations of the use of next-generation sequencing technologies for detecting mutational signatures. DNA Repair (Amst). 2021; 07:103200.
19. Gniadecki R, Iyer A, Hennessey D, Khan L, O'Keefe S, Redmond D, Storek J, Durand C, Cohen-Tervaert JW, Osman M. Genomic instability in early systemic sclerosis. J Autoimmun. 2022; 131:102847.
20. Gaulin C, Kelemen K, Arana Yi C. Molecular pathways in clonal hematopoiesisfrom the acquisition of somatic mutations to transformation into hematologic neoplasm. Life (Basel). 2022; 12:1135.
21. Gusdorf L, Lipsker D. Schnitzler syndrome: a review. Curr Rheumatol Rep. 2017; 19:46.
22. de Koning HD. Schnitzler's syndrome: lessons from 281 cases. Clin Transl Allergy. 2014; 4:41.
23. Treon SP, Xu L, Yang G *et al.* MYD88 L265P somatic mutation in Waldenström's macroglobulinemia. N Engl J Med. 2012; 367:826-833.
24. Del-Rey M, Ruiz-Contreras J, Bosque A, Calleja S, Gomez-Rial J, Roldan E, Morales P, Serrano A, Anel A, Paz-Artal E, Allende LM. A homozygous Fas ligand gene mutation in a patient causes a new type of autoimmune lymphoproliferative syndrome. Blood. 2006; 108:1306-1312.
25. Savola P, Kelkka T, Rajala H, *et al.* Somatic mutations in clonally expanded cytotoxic T lymphocytes in patients with newly diagnosed rheumatoid arthritis. Nat Commun. 2017; 8:15869.
26. Savola P, Brück O, Olson T, Kelkka T, Kauppi MJ, Kovanen PE, Kytölä S, Sokka-Isler T, Loughran TP, Leirisalo-Repo M, Mustjoki S. Somatic *STAT3* mutations

in Felty syndrome: an implication for a common pathogenesis with large granular lymphocyte leukemia. Haematologica. 2018; 103:304-312.
27. Papa R, Caorsi R, Volpi S, Gattorno M. New monogenic autoinflammatory diseases: 2021 year in review. Immunol Lett. 2022; 248:96-98.
28. Louvrier C, Assrawi E, El Khouri E. NLRP3- associated autoinflammatory diseases: henotypic and molecular characteristics of germline versus somatic mutations. J Allergy Clin Immunol. 2020; 145:1254-1261.
29. Omoyinmi E, Melo Gomes S, Standing A. Brief report: Whole-exome sequencing revealing somatic NLRP3 mosaicism in a patient with chronic infantile neurologic, cutaneous, articular syndrome. Arthritis Rheumatol. 2014; 66:197-202.
30. Alcedo PE, Gutierrez-Rodrigues F, Patel BA. Somatic mutations in VEXAS Syndrome and Erdheim-Chester disease: Inflammatory myeloid diseases. Semin Hematol. 2022; 59:156-166.
31. Abla O, Weitzman S. Treatment of Langerhans cell histiocytosis: Role of BRAF/MAPK inhibition. Hematology Am Soc Hematol Educ Program. 2015; 2015:565-570.
32. Ogawa H, Kuroda T, Inada M, Yamamoto M, Enomoto H, Kishima Y, Yoshida K, Ito H, Ogawa H, Nakamura H. Intestinal Behçet's disease associated with myelodysplastic syndrome with chromosomal trisomy 8--a report of two cases and a review of the literature. Hepatogastroenterology. 2001; 48:416-420.
33. Oh E, Akopian D, Rape M. Principles of ubiquitin-dependent signaling. Annu Rev Cell Dev Biol. 2018; 34:137-162.
34. Rape M. Ubiquitylation at the crossroads of development and disease. Nat Rev Mol Cell Biol. 2018; 19:59-70.
35. Aksentijevich I, Zhou Q. NF-kappaB pathway in autoinflammatory diseases: dysregulation of protein modifications by ubiquitin defines a new category of autoinflammatory diseases. Front Immunol. 2017; 8:399.
36. Schulman BA, Harper JW. Ubiquitin-like protein activation by E1 enzymes: The apex for downstream signalling pathways. Nat Rev Mol Cell Biol. 2009; 10:319-331.
37. Lacombe V, Prevost M, Bouvier A, Thépot S, Chabrun F, Kosmider O, Lacout C, Beucher A, Lavigne C, Geneviève F, Urbanski G. Vacuoles in neutrophil precursors in VEXAS syndrome: diagnostic performances and threshold. Br J Haematol. 2021; 195:286-289.
38. Arlet JB, Terrier B, Kosmider O. Mutant UBA1 and severe adult-onset autoinflammatory disease. N Engl J Med. 2021; 384:2163.
39. Grayson PC, Patel BA, Young NS. VEXAS syndrome. Blood. 2021; 137:3591-3594.
40. Huang H, Zhang W, Cai W, Liu J, Wang H, Qin T, Xu Z, Li B, Qu S, Pan L, Huang G, Gale RP, Xiao Z. VEXAS syndrome in myelodysplastic syndrome with autoimmune disorder. Exp Hematol Oncol. 2021; 10: 23.
41. Stubbins RJ. Lost in translation: Cytoplasmic UBA1 and VEXAS syndrome. Blood. 2022; 140:1455-1457.
42. Stubbins RJ, McGinnis E, Johal B, Chen LY, Wilson L, Cardona DO, Nevill TJ. VEXAS syndrome in a female patient with constitutional 45,X (Turner syndrome). Haematologica. 2022; 107:1011-1013.
43. Barba T, Jamilloux Y, Durel CA, Bourbon E, Mestrallet F, Sujobert P, Hot A. VEXAS syndrome in a woman. Rheumatology (Oxford). 2021; 60:e402-e403.
44. Grayson PC, Beck DB, Ferrada MA, Nigrovic PA, Kastner DL. VEXAS syndrome and disease taxonomy in rheumatology. Arthritis Rheumatol. 2022; 74:1733-1736.
45. D'Angelo G. Hematopoietic cells vacuolation, not always a reactive event. The VEXAS syndrome. Int J Lab Hematol. 2023; 45:e15-e16.
46. Uchino K, Kanasugi J, Enomoto M, Kitamura F, Tsuchida N, Uchiyama Y, Maeda A, Kirino Y, Matsumoto N, Takami A. VEXAS syndrome. Int J Hematol. 2022; 116:463-464.
47. Sharma A, Naidu G, Deo P, Beck DB. VEXAS syndrome with systemic lupus erythematosus: Expanding the spectrum of associated conditions. Arthritis Rheumatol. 2022; 74:369-371.
48. Delplanque M, Aouba A, Hirsch P, *et al.* USAID associated with myeloid neoplasm and VEXAS syndrome: Two differential diagnoses of suspected adult onset Still's disease in elderly patients. J Clin Med. 2021; 10:5586.
49. Oganesyan A, Jachiet V, Chasset F, Hirsch P, Hage-Sleiman M, Fabiani B, Duriez P, Georgin-Lavialle S, Delhommeau F, Hakobyan Y, Fain O, Mekinian A; on behalf MINHEMON. VEXAS syndrome: Still expanding the clinical phenotype. Rheumatology (Oxford). 2021; 60:e321-e323.
50. Pyo JY, Ahn SS, Song JJ, Park YB, Lee SW. The reclassification of patients with previously diagnosed eosinophilic granulomatosis with polyangiitis based on the 2022 ACR/EULAR criteria for ANCA-associated vasculitis. J Rheumatol. 2023; 50:213-218.
51. Koster MJ, Kourelis T, Reichard KK, *et al.* Clinical heterogeneity of the VEXAS syndrome: A case series. Mayo Clin Proc. 2021; 96:2653-2659.
52. Ciferska H, Gregová M, Klein M, Šenolt L, Soukupová Maaloufová J, Pavelka K, Mann H. VEXAS syndrome: A report of three cases. Clin Exp Rheumatol. 2022; 40:1449.
53. Khitri MY, Guedon AF, Georgin-Lavialle S, *et al*. French VEXAS group and MINHEMON. Comparison between idiopathic and VEXAS-relapsing polychondritis: Analysis of a French case series of 95 patients. RMD Open. 2022; 8:e002255.
54. Staels F, Betrains A, Woei-A-Jin FJSH, Boeckx N, Beckers M, Bervoets A, Willemsen M, Neerinckx B, Humblet-Baron S, Blockmans DE, Vanderschueren S, Schrijvers R. Case report: VEXAS syndrome: From mild symptoms to life-threatening macrophage activation syndrome. Front Immunol. 2021; 12:678927.
55. Oganesyan A, Hakobyan Y, Terrier B, Georgin-Lavialle S, Mekinian A. Looking beyond VEXAS: Coexistence of undifferentiated systemic autoinflammatory disease and myelodysplastic syndrome. Semin Hematol. 2021; 58:247-253.
56. van der Made CI, Potjewijd J, Hoogstins A, *et al.* Adult-onset autoinflammation caused by somatic mutations in UBA1: A Dutch case series of patients with VEXAS. J Allergy Clin Immunol. 2022; 149:432-439.e4.
57. Muratore F, Marvisi C, Castrignanò P, Nicoli D, Farnetti E, Bonanno O, Longo R, Zaldini P, Galli E, Balanda N, Beck DB, Grayson PC, Pipitone N, Boiardi L, Salvarani C. VEXAS syndrome: A case series from a single-center cohort of Italian patients with vasculitis. Arthritis Rheumatol. 2022; 74:665-670.
58. Bourbon E, Heiblig M, Gerfaud Valentin M, Barba T, Durel CA, Lega JC, Barraco F, Sève P, Jamilloux Y, Sujobert P. Therapeutic options in VEXAS syndrome: insights from a retrospective series. Blood. 2021; 137:3682-3684.
59. Tsuchida N, Kunishita Y, Uchiyama Y, Kirino Y, Enaka M, Yamaguchi Y, Taguri M, Yamanaka S, Takase-Minegishi K,

Yoshimi R, Fujii S, Nakajima H, Matsumoto N. Pathogenic *UBA1*variants associated with VEXAS syndrome in Japanese patients with relapsing polychondritis. Ann Rheum Dis. 2021; 80:1057-1061.
60. Ferrada MA, Savic S, Cardona DO, *et al.* Translation of cytoplasmic UBA1 contributes to VEXAS syndrome pathogenesis. Blood. 2022; 140:1496-1506.
61. Georgin-Lavialle S, Terrier B, Guedon AF, *et al.* French VEXAS group; GFEV, GFM, CEREMAIA, MINHEMON. Further characterization of clinical and laboratory features in VEXAS syndrome: Large-scale analysis of a multicentre case series of 116 French patients. Br J Dermatol. 2022; 186:564-574.
62. Itagane M, Teruya H, Kato T, Tsuchida N, Maeda A, Kirino Y, Uchiyama Y, Matsumoto N, Kinjo M. Clinical images: VEXAS syndrome presenting as treatment-refractory polyarteritis nodosa. Arthritis Rheumatol. 2022; 74:1863-1864.
63. Matsumoto H, Asano T, Tsuchida N, *et al.* Behçet's disease with a somatic UBA1 variant:Expanding spectrum of autoinflammatory phenotypes of VEXAS syndrome. Clin Immunol. 2022; 238:108996.
64. Watanabe R, Kiji M, Hashimoto M. Vasculitis associated with VEXAS syndrome: A literature review. Front Med (Lausanne). 2022; 9:983939.
65. Vitale A, Caggiano V, Della Casa F, *et al.* Development and implementation of the AIDA international registry for patients with VEXAS syndrome. Front Med (Lausanne). 2022; 9:926500.
66. Topilow JS, Ospina Cardona D, Beck DB, Ferrada MA, McMahan ZH, Paik JJ. Novel genetic mutation in myositis-variant of VEXAS syndrome. Rheumatology (Oxford). 2022; 61:e371-e373.
67. Vergneault H, Picard C, Georgin-Lavialle S. Break down the barriers of auto-inflammation: How to deal with a monogenic auto-inflammatory disease and immuno-haematological features in 2022? Immunology. 2023; 168:1-17.
68. Lacombe V, Kosmider O, Prévost M, Lavigne C, Urbanski G. Severe joint involvement in VEXAS syndrome: A case report. Ann Intern Med. 2021; 174:1025-1027.
69. McHugh J. Pathogenic UBA1 variants define a subset of relapsing polychondritis. Nat Rev Rheumatol. 2021; 17:312.
70. Luzzatto L, Risitano AM, Notaro R. Mutant UBA1 and severe adult-onset autoinflammatory disease. N Engl J Med. 2021; 384:2164.
71. Zakine E, Schell B, Battistella M, *et al.* UBA1 Variations in neutrophilic dermatosis skin lesions of patients with VEXAS syndrome. JAMA Dermatol. 2021; 157:1349-1354.
72. Kirino Y, Takase-Minegishi K, Tsuchida N, Hirahara L, Kunishita Y, Yoshimi R, Nakajima H. Tocilizumab in VEXAS relapsing polychondritis: A single-center pilot study in Japan. Ann Rheum Dis. 2021; 80:1501-1502.
73. Goyal A, Narayanan D, Wong W, Laga AC, Connell NT, Ritter SY, Cobos G. Tocilizumab for treatment of cutaneous and systemic manifestations of vacuoles, E1 enzyme, X-linked, autoinflammatory, somatic (VEXAS) syndrome without myelodysplastic syndrome. JAAD Case Rep. 2022; 23:15-19.
74. Kunishita Y, Kirino Y, Tsuchida N, Maeda A, Sato Y, Takase-Minegishi K, Yoshimi R, Nakajima H. Case Report: Tocilizumab treatment for VEXAS syndrome with relapsing polychondritis: A single-center, 1-year longitudinal observational study in Japan. Front Immunol. 2022; 13:901063.
75. Guilpain P. JAK inhibitors in autoinflammatory syndromes? The long road from drug development to daily clinical use. Rheumatology (Oxford). 2023; 62:1368-1369.
76. Heiblig M, Patel BA, Groarke EM, Bourbon E, Sujobert P. Toward a pathophysiology inspired treatment of VEXAS syndrome. Semin Hematol. 2021; 58:239-246.
77. Fu Y, Wu W, Chen Z, Gu L, Wang X, Ye S. Trisomy 8 associated clonal cytopenia featured with acquired auto-inflammation and its response to JAK inhibitors. Front Med (Lausanne). 2022; 9:895965.
78. Conway R. Ruxolitinib takes center stage for VEXAS syndrome. Blood. 2022; 140:807-808.
79. Heiblig M, Ferrada MA, Koster MJ, *et al.* Ruxolitinib is more effective than other JAK inhibitors to treat VEXAS syndrome: A retrospective multicenter study. Blood. 2022; 140:927-931.
80. Magnol M, Couvaras L, Degboé Y, Delabesse E, Bulai-Livideanu C, Ruyssen-Witrand A, Constantin A. VEXAS syndrome in a patient with previous spondyloarthritis with a favourable response to intravenous immunoglobulin and anti-IL17 therapy. Rheumatology (Oxford). 2021; 60:e314-e315.
81. Cordts I, Hecker JS, Gauck D, Park J, Härtl J, Günthner R, Hammitzsch A, Schoser B, Abeck D, Götze KS, Haack TB, Deschauer M, Moog P, Hemmer B. Successful treatment with azacitidine in VEXAS syndrome with prominent myofasciitis. Rheumatology (Oxford). 2022; 61:e117-e119.
82. Raaijmakers MHGP, Hermans M, Aalbers A, Rijken M, Dalm VASH, van Daele P, Valk PJM. Azacytidine treatment for VEXAS syndrome. Hemasphere. 2021; 5:e661.
83. Manzoni M, Bosi A, Fabris S, *et al.* Clinical, morphological and clonal progression of VEXAS syndrome in the context of myelodysplasia treated with azacytidine. Clin Hematol Int. 2022; 4:52-55.
84. Comont T, Heiblig M, Rivière E, *et al.* Azacitidine for patients with Vacuoles, E1 Enzyme, X-linked, Autoinflammatory, Somatic syndrome (VEXAS) and myelodysplastic syndrome: Data from the French VEXAS registry. Br J Haematol. 2022; 196:969-974.
85. Al-Hakim A, Poulter JA, Mahmoud D, Rose AMS, Elcombe S, Lachmann H, Cargo C, Duncan CJA, Bishton M, Bigley V, Khan A, Savic S. Allogeneic haematopoietic stem cell transplantation for VEXAS syndrome: UK experience. Br J Haematol. 2022; 199:777-781.
86. Diarra A, Duployez N, Fournier E, *et al.* Successful allogeneic hematopoietic stem cell transplantation in patients with VEXAS syndrome: A 2-center experience. Blood Adv. 2022; 6:998-1003.

Received March 1, 2023; Revised July 30, 2023; Accepted August 15, 2023.

**Address correspondence to:*
Senol Kobak, Department of Internal Medicine and Rheumatology, Istinye University Faculty of Medicine, Liv Hospital, WASOG Sarcoidosis Clinic, Canan sokak No:5 Istanbul, Turkey.
E-mail: senolkobak@yahoo.com

Released online in J-STAGE as advance publication August 18, 2023.

Original Article

DOI: 10.5582/irdr.2023.01050

The novel role of IFITM1–3 in myogenic differentiation of C2C12 cells

Yongtao Zhang, Yanqin Lu*, Xianxian Li, Shanshan Zhang, Pengchao Liu, Xiaoyang Hao, Jinxiang Han*

Key Laboratory for Biotech Drugs of the National Health Commission, Key Laboratory for Rare & Uncommon Diseases of Shandong Province, Biomedical Science College & Shandong Medicinal Biotechnology Centre, Shandong First Medical University & Shandong Academy of Medical Sciences, Ji'nan, Shandong, China.

SUMMARY Interferon-induced transmembrane proteins (IFITMs 1, 2, and 3) play a critical role in preventing pathogen infection in vertebrates. They are also involved in the occurrence and prognosis of cancer. Myogenesis is a complex process regulated by several factors. This study disclosed that Ifitm1–3 were upregulated in the process of myogenic differentiation of C2C12 myoblasts on days 3, 5, and 7. This positively correlated with the expression of differentiation factors MyoD, myogenin, Mrf5, and desmin. Furthermore, knockdown of Ifitm1–3 by their individual siRNAs inhibited myogenesis of C2C12 myoblasts, with relative downregulation of MyoD, myogenin, Mrf5, and desmin. Subsequently, myotube formation and fusion percentage decreased. Co-immunoprecipitation combined with LC-MS/MS analysis uncovered the interaction proteins of IFITM1 and IFITM3 in C2C12 myoblasts. A total of 84 overlapped interaction proteins of IFITM1 and IFITM3 were identified, and one of the clusters was engaged in cytoskeletal and sarcomere proteins, including desmin, myosin, actin, vimentin, nestin, ankycorbin, and nucleolin. Hence, we hypothesize that these interacting proteins may function as scaffolds for IFITM1–3, possibly through the interaction protein desmin to initiate further interaction with other proteins to participate in myogenesis; however, the molecular mechanisms remain unclear. Our study may contribute to the development of novel therapeutics for myopathic diseases.

Keywords IFITM1, IFITM3, myogenesis, desmin, sarcomere

1. Introduction

The interferon-inducible transmembrane proteins (IFITMs) belong to the small interferon-stimulated family with molecular mass ranging from 10 to 20 kDa (*1*). The members of this family include IFITM1, IFITM2, IFITM3, IFITM5, and IFITM10 in *Homo sapiens*, and IFITM1, IFITM2, IFITM3, IFITM5, IFITM6, and IFITM10 in mice (*1,2*). IFITM expression can be regulated dependently or independently of interferon signaling (*3-5*).

IFITM1–3 play a synergistic role in antiviral defense, and they are key participants in antiviral immunity (*6-9*). Their expression is upregulated by interferons and other cytokines. When expressed on the cell membrane, they can limit virus–cell membrane fusion, thereby preventing virus entry into the cytoplasm (*10*). IFITM1–3 also suppress viral protein synthesis or viral replication, leading to the reduced production of infectious virions or the lower infectivity of offspring virions (*1,11-13*). IFITM1–3 are also responsible for germ cell homing and maturation during embryonic development (*14,15*). They inhibit placental syncytiotrophoblast fusion (*16-18*). The expression of IFITM5 gradually increases with the differentiation of osteoblasts, and it is an important positive regulator in osteogenic differentiation (*19,20*). *IFITM5* gene mutations lead to type V osteogenesis imperfecta (*21,22*). Low expression of IFITM10 has been reported in re-set positive patients with SARS-CoV-2 infection, and IFITM10 expression level positively correlates with the expression levels of CD69, CD44, NKp30, and granzyme B (*23*). Recently, IFITMs have been implicated in cancer cell progression *via* different pathways (*24-32*).

The process of myogenic differentiation includes the activation, proliferation, and differentiation of resting satellite cells into myoblasts, which fuse to form myotubes, and ultimately mature into muscle fibers that

contain cytoskeletal proteins actin, myosin, desmin, and vimentin, and differentiation factors MyoD, mrf5, MRF4, and myogenin (*33-36*). In this study, we explored the expression of IFITM1–3 in the process of myogenesis and their interaction proteins in myoblasts.

2. Materials and Methods

2.1. Cell culture and myogenic differentiation of C2C12

C2C12 (ATCC) cells were cultured in high-glucose Dulbecco's modified Eagle medium (DMEM) (Lonza, Switzerland) supplemented with 10% (v/v) fetal bovine serum (Gibco, America), 2 mM glutamine (Gibco, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco, USA) (growth medium). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. To induce myogenic differentiation, C2C12 cells were grown to 70–80% confluence, with 5×10^4 cells seeded in six-well plates, and then the media were switched to low serum differentiation media (Gibco, USA) (DMEM supplemented with 5% (v/v) horse serum (Gibco, USA) and penicillin/ streptomycin). Fresh differentiation media were changed every 24 h until the end of the assay.

2.2. siRNA targeting *Ifitm1–3*

Small-interfering RNA directed against the mice *Ifitm1–3* was designed by siDirect (*http://sidirect2.rnai.jp/*) and was synthesized by GenePharma (Shanghai, China). ExFect transfection reagent (Vazyme, Nanjing, China), siRNAs, and negative controls (si-NC) were used to transfect C2C12 cells for 6 h. The cells were transfected using ExFect transfection reagent (Vazyme, Nanjing, China) following the manufacturer's instructions. Cells transfected with transfection reagent only were used as the mock control. Then, the supernatant was replaced by myogenic differentiation media as described above. The designed sense strand of siRNA was as follows: si1-*Ifitm1*: 5′-GCAGCAAGAGGUGGUUGUATT-3′; si2-*Ifitm1*: 5′-CAAGCUAUGAGACAAUCAATT-3′; si1-*Ifitm2*: 5′-GACAAUCAAAGAGGAGUATT-3′; si2-*Ifitm2*: 5′-GGGUCACCCCACAUCUCAATT-3′; si1-*Ifitm3*: 5′-CGAAAGAAUCAAGGAAGAATT-3′; si2-*Ifitm3*: GUUGUUAUCACCAUUGUUATT-3′.

2.3. RNA extraction and RT-qPCR

Total RNA from C2C12 cultured in differentiation and un-differentiation medium was isolated using FastGene RNA Basic Kit (Takara, Japan) in accordance with the manufacturer's instructions. RNA purity and integrity were evaluated using a NanoDrop-2000 spectrophotometer. After DNase I (Takara, Japan) digestion, total RNAs were reverse-transcribed to cDNA using ReverTra Ace qPCR RT Kit (Takara, Japan).

Table 1. Primer sequences for RT-qPCR

Primer	Primer sequence (5′-3′)
Mus-Ifitm1	F: GCTCCTCGACCACACCTCT
	R: TGGAGATCTCAGGCATGTTG
Mus-Ifitm2	F: TGGGCTTCGTTGCCTATGC
	R: AGAATGGGGTGTTCTTTGTGC
Mus-Ifitm3	F: CCCCCAAACTACGAAAGAATCA
	R: ACCATCTTCCGATCCCTAGAC
Mus-desmin	F: GTGAAGATGGCCTTGGATGT
	R: AAGGTCTGGATCGGAAGGTT
Mus-MyoD	F: AGCACTACAGTGGCGACTCA
	R: GGCCGCTGTAATCCATCAT
Mus-Myf5	F: CTGCTCTGAGCCCACCAG
	R: GACAGGGCTGTTACATTCAGG
Mus-myogenin	F: GAGACATCCCCCTATTTCTACCA
	R: GCTCAGTCCGCTCATAGCC
Mus-Gapdh	F: CATCCCAGAGCTGAACG
	R: CTGGTCCTCAGTGTAGCC

qPCR was performed using 2× SYBR Green qPCR Mix (SparkJade, Bio, China) with a Lightcycler 480. The PCR program was as follows: initial 5 min denaturation at 95°C, followed by 45 cycles of amplification at 95°C for 10 s, 60°C for 10 s, and 72°C for 15 s. To quantify the expression of each candidate gene, the mRNA expression levels were normalized to the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. Relative gene expression was calculated with the $2^{-\Delta\Delta Ct}$ method. Each sample was performed in triplicate. Sequences of the forward and reverse primers for RT-qPCR are shown in Table 1.

2.4. Western blotting

C2C12 cells in the six-well plate were washed with precooled phosphate buffer solution (PBS) and lysed with 200 μL of protein RIPA lysis buffer supplemented with 1% PMSF (CWBIO, China) per well for 1 h on ice. Then, the extracted precipitate was quantified using the BCA protein concentration assay kit (Biosharp, China). The processed protein samples (20 μg/line) were resolved using 12% SDS-PAGE at 60 V in the stacking gel and at 120 V in the separating gel. Proteins in the gels were transferred to polyvinylidene fluoride membranes (0.45 μm, Biosharp, China). The membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 2 h at room temperature, and then the membranes were incubated with primary antibodies, namely, rabbit anti-desmin (ab32362, Abcam, USA), rabbit anti-MyoD (ab203383, Abcam, USA), rabbit anti-MYF5 (ab125301, Abcam, USA), rabbit anti-IFITM1 (bs-1031R, Bioss, China), rabbit anti-IFITM2 (bs-15517R, Bioss, China), mouse anti-IFITM3 (bsm-51629M, Bioss, China), and rabbit anti-GAPDH (ab8245, Abcam, USA), at 4°C overnight. Then, the membranes were incubated in PBS-Tween containing anti-rabbit-horseradish peroxidase–conjugated secondary antibodies (SA00001, Proteintech, USA) for

1 h at 37°C. The membranes were washed in PBST, and signals of the protein blots were acquired using an ECL Chemiluminescence Substrate Kit (Biosharp, China) and visualized by exposing the membranes in a Chemiluminescence Gel Imaging System (18200880, Alliance, UK). The level of expression of each protein was normalized to that of GAPDH. The results were quantified using ImageJ-win64 software (Rawak Software Inc., Stuttgart, Germany).

2.5. Giemsa staining

C2C12 cells were gently washed with PBS three times and fixed with anhydrous methanol for 15 min. The Giemsa staining solutions were diluted with PBS. C2C12 cells were then incubated with the diluted staining solution for 15 min at 37°C and washed with PBS twice. Morphological observation was conducted using an inverted microscope. Images were taken randomly of three different sections per dish. The number of nuclei in myotubes and the total number of nuclei in the cells were counted in each field. Fusion indexes were calculated by expressing the number of nuclei in the myotubes as percentages of the total number of nuclei.

2.6. Immunoprecipitation

Co-immunoprecipitation was performed using the Pierce™ Classic Magnetic IP/Complex Immunoprecipitation (Co-IP) Kit (88804, Thermo, USA) in accordance with the manufacturer's protocol. Briefly, C2C12 cells were lysed with RIPA lysis buffer supplemented with 1% PMSF as described above. Then, 200 μL of the cell lysate was incubated with a mixture of 20 μL of protein A/G beads and 5 μL of conjugated antibody to IFITM1 or IFITM3 overnight with rotation at 4°C. The beads were then washed with IP (Immunoprecipitation) lysis/wash buffer and PBS. An aliquot of 200 μL of elution buffer was added to the beads, which were incubated at room temperature and mixed for 5–10 min. The supernatant was transferred to a new tube. Finally, the samples were loaded onto SDS-PAGE gel for LC-MS/MS analysis or western blot analysis.

2.7. LC-MS/MS analysis

Proteins in gel pieces were destained in 50 mM NH_4HCO_3 in 50% acetonitrile (v/v) until clear. Gel pieces were dehydrated with 100 μL of 100% acetonitrile for 5 min; the liquid was removed; and the gel pieces were rehydrated in 10 mM dithiothreitol and incubated at 56°C for 60 min. The gel pieces were again dehydrated in 100% acetonitrile; the liquid was removed; and the gel pieces were rehydrated with 55 mM iodoacetamide. The samples were incubated at room temperature for 45 min in the dark. The gel pieces were washed with 50 mM NH_4HCO_3 and dehydrated with 100% acetonitrile. They were rehydrated with 10 ng/μL trypsin resuspended in 50 mM NH_4HCO_3 on ice for 1 h. Excess liquid was removed, and the gel pieces were digested with trypsin at 37°C overnight. Peptides were extracted with 50% acetonitrile/5% formic acid, followed by 100% acetonitrile. Peptides were dried to completion and resuspended in 2% acetonitrile/0.1% formic acid.

The tryptic peptides were dissolved in 0.1% formic acid (solvent A) and directly loaded onto a home-made reversed-phase analytical column (15-cm length, 75 μm i.d.). The gradient comprised an increase from 6% to 23% solvent B (0.1% formic acid in 98% acetonitrile) over 16 min, a rise from 23% to 35% in 8 min, and climbing to 80% in 3 min, followed by holding at 80% for the last 3 min, all at a constant flow rate of 400 nL/min on an EASY-nLC 1000 UPLC system.

The peptides were subjected to NSI source followed by tandem mass spectrometry (MS/MS) in Q ExactiveTM Plus (Thermo) coupled online to the UPLC. The electrospray voltage applied was 2.0 kV. The m/z scan range was 350 to 1,800 for full scan, and intact peptides were detected in the Orbitrap at a resolution of 70,000. Peptides were then selected for MS/MS using NCE setting as 28 and the fragments were detected in the Orbitrap at a resolution of 17,500. A data-dependent procedure alternated between one MS scan followed by 20 MS/MS scans with 15.0 s dynamic exclusion. Automatic gain control (AGC) was set at 5E4.

The peptides were detected, isolated, and fragmented to produce a tandem mass spectrum of specific fragment ions for each peptide. The resulting MS/MS data were processed using Proteome Discoverer 1.3 and Uniprot database (*https://www.uniprot.org*).

2.8. Protein–protein interaction, KEGG, and GO enrichment analysis

A Venn diagram was drawn (*http://bioinformatics.psb.ugent.be/webtools/Venn*) to conduct an intersection analysis to compare binding proteins of IFITM1 and IFTM3. Protein–protein interaction was conducted by online String analysis (*https://string-db.org/*). A high confidence of 0.700 was set as the required score, and stringency was set as high (1%). Protein–protein interaction was clustered by kmeans. In addition, we performed Gene Ontology (GO) analysis. First, the data of function annotation diagram were obtained using the DAVID website (*https://david.ncifcrf.gov*), and the data with $P < 0.05$ were selected; the enriched paths were displayed using "tidyr" and "ggplot2" R packages. R package "cluster profiler" was used for GO enrichment analysis. Using the cnetplot function (circular = F, color edge = T, node tag = T), the data of GO analysis can be visualized as cnetplot. Data processing and mapping were performed using R-project (v4.0.5) and Rstudio

software (v1.3.1093).

2.9. Statistical analysis

The results are presented as the mean ± standard error of the mean (SEM). Statistical comparisons were made based on one-way ANOVA and Tukey's multiple-comparison test using GraphPad Prism software, version 7.0 (GraphPad Software Inc., San Diego, CA, USA), to)identify significant differences. P values < 0.05 were considered statistically significant (* represents $P < 0.05$, ** represents $P < 0.01$, *** represents $P < 0.001$). All experiments were performed at least three times.

3. Results

3.1. The increased expression of IFITMs in myogenic differentiation of C2C12 cells

The essential step in myogenesis is cell fusion and the formation of myotubes, whereby mononuclear myocytes fuse to form multinucleated myotubes. As expected, the number of fusing myocytes and myotubes increased on the subsequent induction day (Figure 1A and 1B). Gene and protein expression levels of desmin, Myf5, myogein, and MyoD on days 3, 5, and 7 after myogenic differentiation induction were consistent and significantly increased (Figure 1C–E).

The results of RT-PCR analysis indicated that the expression levels of *Ifitm1–3* increased during the differentiation of C2C12 cells. The differentiation medium stimulated the expression of *Ifitm1–3*, with the higher levels observed on the third day and thereafter (Figure 2A). Overall trends showed an increase in the expression of IFITM1–3 proteins in the differentiation process of C2C12 cells, with the higher values on days 3 and 7 compared with that on day 0 before myogenic induction. For IFITM1, decreased expression was observed on day 5, compared with day 3, and then climbed to the highest value on day 7. Protein level of IFITM2 was higher on days 3, 5, and 7 than on day 0 in the process of myogenic differentiation. IFITM3 expression was not significantly increased on day 5 compared with day 3, but there was an obvious increasing trend on day 7 (Figure 2B–C).

3.2. si-RNA knocks down the expression of *Ifitm1–3* in myogenic differentiation of C2C12 cells

Theoretically, the upregulated *Ifitm1–3* during the process of myogenic induction could be blocked by targeting *Ifitms* with small-interfering RNA (siRNA). Hence, we initially screened the siRNAs with high inhibition efficiency for targeting *Ifitm1–3*. The interference efficiency of siRNAs was determined to be over 70% as identified by RT-qPCR (Supplementary Figure S1, *http://www.irdrjournal.com/action/getSupplementalData.php?ID=152). Myotube formation was also evaluated*

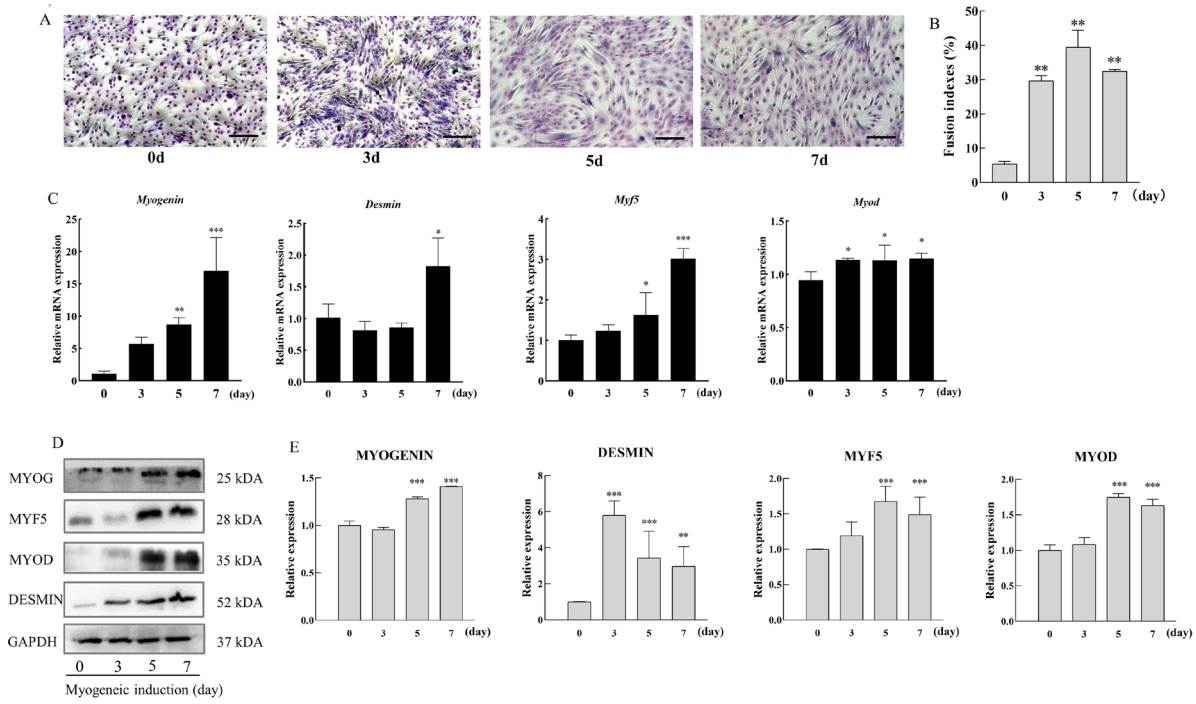


Figure 1. Myogenic differentiation of C2C12 myoblasts on days 0, 3, 5, and 7. (A) Microscopic images of C2C12 cells (stained by Giemsa staining prior to induction) and myoblasts. Magnification: 100×. **(B)** Relative expression of myogenin, myogenic differentiation factor D (MyoD), Myf5, and desmin mRNA as measured by qRT-PCR. **(C)** Representative bands for myogenin, MyoD, Myf5, desmin, and GAPDH are shown. **(D)** Quantification of band intensity as described above is shown. The level of proteins was normalized to that of GAPDH. Statistical significance: *P <0.05; **P <0.01; ***P <0.001; NS, not significant.

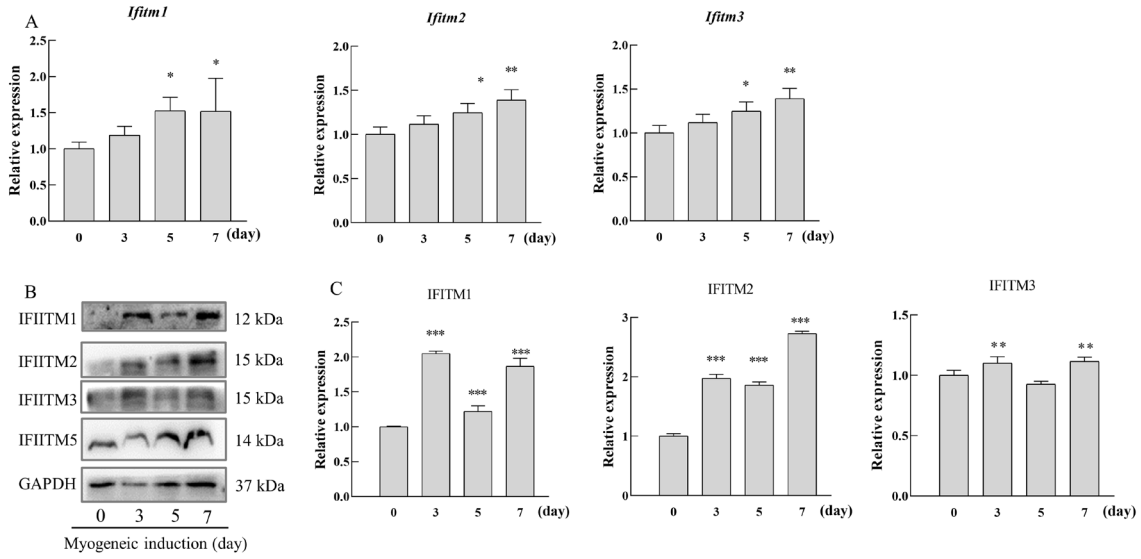


Figure 2. Increased expression of IFITM1–3 during myogenic differentiation of C2C12 myoblasts. (A) Relative expression of *Ifitm1–3* during the myogenic differentiation process. (B) Western blot evaluating the protein levels of IFITM1–3. (C) Quantification of band intensity as described above is shown. The level of proteins was normalized to that of GAPDH. Statistical significance: **P* < 0.05; ***P* < 0.01; ****P* < 0.001; NS, not significant.

*as the f*usion index. By the transfection of specific siRNA1 and siRNA2 targeting *Ifitm1–3* respectively, the percentage of fusion index decreased significantly in the interference groups of *Ifitm1–3*, compared with Mock group (Figure 3A–B). Especially in the *si2-Ifitm1* group, the percentage of fusion index decreased to 14.82% (*P* < 0.01%) (Figure 3B). Consistent with that, the expression of MyoD, myogenin, MYF5, and desmin was significantly repressed with differentiation in the *Ifitm1–3* knockdown groups as shown by western blot (Figure 3C–D).

3.3. Interacting proteins of IFITMs and GO enrichment analysis

In order to determine the interaction proteins of IFITM1–3, we performed immunoprecipitation–mass spectrometry (IP-MS) assays using IFITM1 and IFITM3 antibodies. Specific bands identified exclusively in the pull-down products of antibodies were excised for protein identification using MS (Figure 4A). A total of 84 proteins, including myosin, actin, vimentin, desmin, myosin regulatory light chain 12B, tropomyosin, and nucleolin (Table 2), were repeatedly identified in all three biological replicates. String analysis revealed 82 nodes, with an average node degree of 13.1. Clustering analysis identified three distinctive groups as follows: the cluster with green color included proteins involved in the process of muscle filament sliding and constituent proteins of sarcomere; the cluster with red color included ribosomal proteins; and the cluster in blue color included the heterogeneous nuclear ribonucleoprotein, histones, and proteins that regulate mRNA metabolic process (Figure 4B). We further validated and confirmed that desmin identified through IP-MS was indeed a bona fide interacting partner of IFITM1 and IFITM3 (Figure 4C).

The KEGG pathway analysis showed that the genes were mainly enriched in hypertrophic cardiomyopathy, dilated cardiomyopathy, cardiac muscle contraction, adrenergic signaling in cardiomyocytes, mRNA surveillance pathway, and RNA transport pathways (Figure 5A). The GO enrichment analyses revealed 329 biological process (BP) entries, involving ribonucleoprotein complex biogenesis, non-membrane-bounded organelle assembly, ribosome biogenesis, actin filament organization, muscle contraction, ncRNA processing, RNA splicing, and muscle system process (Figure 5B); 124 cell component (CC) entries, involving ribosomal subunit, ribosome, cell leading edge, large and small ribosomal subunit, postsynaptic density, asymmetric synapse, postsynaptic specialization, neuron to neuron synapse, contractile fiber, myofibril, myosin complex, stress fiber, contractile actin filament bundle, actin filament bundle actomyosin, and myosin II complex (Figure 5C); and 71 molecular function (MF) entries, involving structural constituent of ribosome, actin binding, actin filament binding, mRNA binding, rRNA binding, structural constituent of cytoskeleton, and cytoskeletal motor activity (Figure 5D).

4. Discussion

The functions of IFITMs are not sufficiently understood. IFITM1–3 inhibit viral fusion and cell entry with a broad virus spectrum. Our study identified that IFITM1–3 were involved in myogenesis and that they were upregulated in this process. The high expression of *Ifitm1–3* has also been reported in the differentiation of H92C cells, a rat

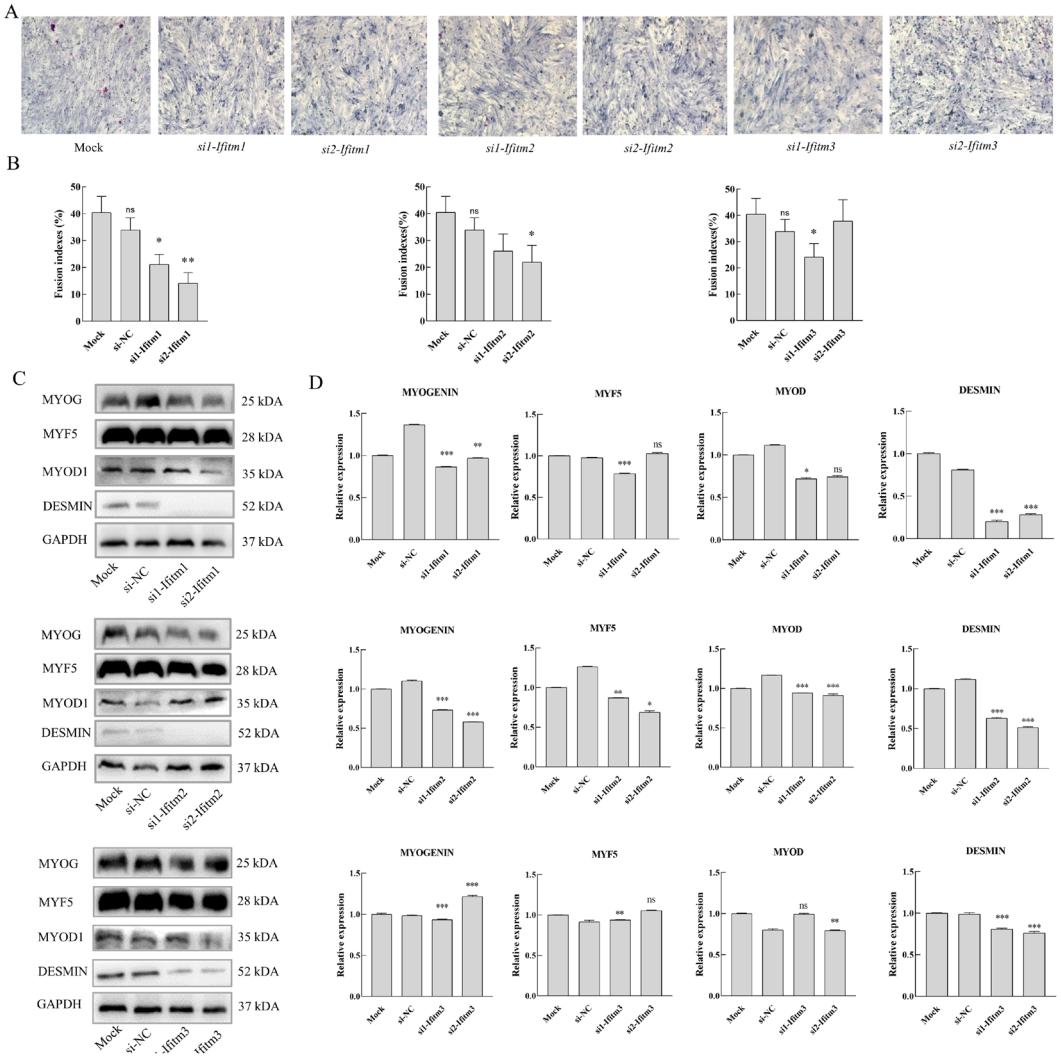


Figure 3. Knockdown of Ifitm1, 2, and 3 by siRNAs blocks myogenic differentiation in C2C12 cells. (A) Microscopic images of Giemsa staining for C2C12 myoblasts on day 3 of myogenic differentiation after transfection with siRNAs targeting *Ifitm1–3*. Two different siRNAs were used for each targeting gene. Transfection without siRNAs, but with transfection reagent was set as mock. Magnification: 100×. (B) Percentage fusion on day 3 of myogenic induction and transfection with siRNAs as described above, calculated by dividing the number of nuclei within multinucleated myofibers by the total number of nuclei. NC represents the group without siRNAs and transfection reagents. (C) Downregulated protein expression of myogenin, MyoD, Myf5, and desmin after interference by siRNAs targeting *Ifitm1–3.* (D) Quantification of band intensity as described above is shown. The level of proteins was normalized to that of GAPDH. Statistical significance: **P*<0.05; ***P* <0.01; ****P* <0.001; NS, not significant.

myoblast cell line (*37*).

IFITMs belong to the CD225 family of proteins. They consist of a conserved intracellular loop between the intramembrane domain and the transmembrane helix (*38,39*). Interaction proteins of IFITMs in different cell lines have been reported, including BRI3, SERINC5, and CAV-1 (*40-42*). All these molecules are related to antiviral effects. In this study, we uncovered that the interacting proteins of IFITM1 and IFITM3 clustered into cytoskeleton proteins ankycorbin, nestin, vimentin, actin, myosin, and desmin.

The sarcomere is the smallest functional unit of muscle fibers in the skeletal muscle, which is arranged between two Z-lines. The constituent proteins of the sarcomere include contractile proteins actin and myosin, structural proteins titin and nebulin, and intermediate filaments (*43*). Notably, we found that IFITM1 and IFITM3 interact with all these cytoskeleton proteins. Desmin encodes a muscle-specific class III intermediate filament and plays an essential role in muscular structure and function. Homopolymers of the protein form a stable intracytoplasmic filamentous network that connects myofibrils to each other and to the plasma membrane (*44*). As one of the earliest markers of an initial step in myogenic differentiation, the expression of desmin precedes the expression of all other muscle-specific structural genes and myogenic helix-loop-helix transcription factors MyoD, myogenin, and Mrf4 (*45*). In desmin-null mutant embryonic stem cell–derived embryoid bodies, skeletal and smooth muscle myogenesis were completely inhibited, displaying the absence of myotube formation, contractility, and expression of MyoD, myogenin, Myf5, and myosin heavy chain (*46*). In desmin-knockout mice, the loss of sarcomere integrity

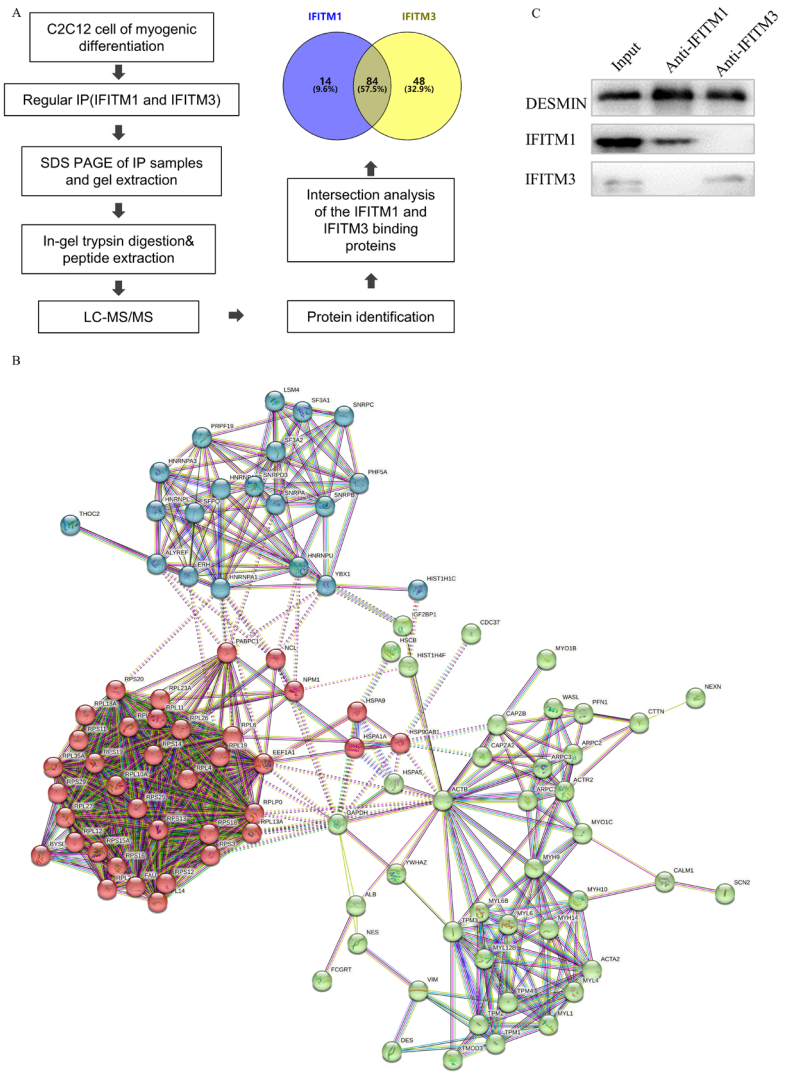


Figure 4. Identification and validation of IFITM1,3-interacting proteins. (A) Principle of co-immunoprecipitation and liquid chromatography combined with tandem mass spectrometry (LC–MS/MS). (B) The protein–protein interaction network if IFITM1,3 (overlapped) revealed by STRING analysis. A total of 84 unique homologous proteins are shown in the network. Three clusters are indicated in different colors. Cluster 1: muscle filament sliding (green color); Cluster 2: ribosome series proteins (red color); Cluster 3: regulation of mRNA metabolic process (blue color). Associations are represented by the lines. Thicker lines represent stronger associations. (C) Co-IP assays show the interaction between desmin and IFITM1, 3.

and the increased number of mitochondria in myofibers were obvious. A reduced gain of muscle performance was observed in mechanical-overload mice that lacked desmin (*47*). Desmin is the pathogenic gene for dilated cardiomyopathy-1I, myofibrillar myopathy-1, and neurogenic scapuloperoneal syndrome (*48-50*). Desmin-null mice demonstrate a multisystem disorder that involves cardiac, skeletal, and smooth muscles at the early stage of their postnatal life (*51*). During murine feto-embryonic development, there is coexpression of desmin and α-actin in skeletal muscle cells, while the expression of desmin precedes that of α-actin in myoblasts of somites (*52*).

Vimentin and nestin also belong to intermediate filaments that participate in the formation of cytoskeleton and maintenance of cell morphology. Vimentin is poorly expressed during myogenic differentiation as it is replaced by the tissue-specific intermediate filament protein, desmin (*53*). Nestin regulates the differentiation of myogenic precursor cells by Cdk5 and it is downregulated in myoblasts (*54,55*). Nestin-knockout mice have a spontaneous regenerative phenotype in skeletal muscle, which is related to a disturbed proliferation cycle under the uncontrolled Cdk5 kinase activity (*54*). Sliding movement between myosin and actin is the molecular basis of muscle contraction. These two proteins are also involved in cytokinesis, locomotion, cell motility, and maintenance of cell shape (*56,57*).

The functions of IFITMs in oncogenesis have been explored in recent years. IFITM3 binds to PIP3 to amplify PIP3 signaling and Src-kinase signaling in B cells by interacting with activated B-cell receptor (BCR) complex. Phosphorylation of Ifitm3-Y20 transfers the location of endosome to the cell surface, thereby initiating the amplification loop of oncogenic signaling (*58*). IFITM3 is involved in gastric cancer, hepatocellular carcinoma, and prostate cancer through the MET/AKT/FOXO3/c-MYC axis, p38/MAPK signaling, and TGF-β signaling pathway, respectively (*30,59,60*).

Our study showed the interaction proteins of IFITMs and their involvement in the myogenic differentiation. Although further studies need to explore the molecular

Table 2. Overlapped interacted proteins for IFITM1 and IFITM3

	Accession	Protein names	Gene names	MW [kDa]
1	Q8VDD5	Myosin-9	*Myh9*	226.23
2	Q61879	Myosin-10	*Myh10*	228.85
3	P60710	Actin, cytoplasmic 1	*Actb*	41.71
4	P62737	Actin, aortic smooth muscle	*Acta2*	41.98
5	P20152	Vimentin	*Vim*	53.66
6	P31001	Desmin	*Des*	53.47
7	Q3THE2	Myosin regulatory light chain 12B	*Myl12b*	19.77
8	Q60605	Myosin light polypeptide 6	*Myl6*	16.92
9	P58774	Tropomyosin beta chain	*Tpm2*	32.82
10	Q6URW6	Myosin-14	*Myh14*	228.45
11	P58771	Tropomyosin alpha-1 chain	*Tpm1*	32.66
12	P09405	Nucleolin	*Ncl*	76.68
13	P21107	Tropomyosin alpha-3 chain	*Tpm3*	32.97
14	Q61937	Nucleophosmin	*Npm1*	32.54
15	P05977	Myosin light chain 1/3, skeletal muscle isoform	*Myl1*	20.58
16	P0DP26	Calmodulin-1	*Calm1*	16.83
17	Q9ERG0	LIM domain and actin-binding protein 1	*Lima1*	84.01
18	Q6IRU2	Tropomyosin alpha-4 chain	*Tpm4*	28.45
19	Q9WTI7	Unconventional myosin-Ic	*Myo1c*	121.87
20	P47757	F-actin-capping protein subunit beta	*Capzb*	31.33
21	P07724	Albumin	*Alb*	68.65
22	Q9EP71	Ankycorbin	*Rai14*	108.79
23	P49312	Heterogeneous nuclear ribonucleoprotein A1	*Hnrnpa1*	34.18
24	Q6P5H2	Nestin	*Nes*	207.00
25	P10126	Elongation factor 1-alpha 1	*Eef1a1*	50.08
26	Q61696	Heat shock 70 kDa protein 1A	*Hspa1a*	70.04
27	Q9CZX8	40S ribosomal protein S19	*Rps19*	16.08
28	P67984	60S ribosomal protein L22	*Rpl22*	14.75
29	P01942	Hemoglobin subunit alpha	*Hba*	15.08
30	P62908	40S ribosomal protein S3	*Rps3*	26.66
31	P62852	40S ribosomal protein S25	*Rps25*	13.73
32	Q60598	Src substrate cortactin	*Cttn*	61.21
33	P62960	Y-box-binding protein 1	*Ybx1*	35.71
34	Q9JHJ0	Tropomodulin-3	*Tmod3*	39.48
35	P35979	60S ribosomal protein L12	*Rpl12*	17.79
36	Q91WK0	Leucine-rich repeat flightless-interacting protein 2	*Lrrfip2*	47.12
37	P20029	Endoplasmic reticulum chaperone BiP	*Hspa5*	72.38
38	P15864	Histone H1.2	*H1-2*	21.25
39	P62862	40S ribosomal protein S30	*Fau*	6.64
40	Q9CR57	60S ribosomal protein L14	*Rpl14*	23.55
41	P29341	Polyadenylate-binding protein 1	*Pabpc1*	70.63
42	P62270	40S ribosomal protein S18	*Rps18*	17.71
43	Q8CI43	Myosin light chain 6B	*Myl6b*	22.73
44	Q8VIJ6	Splicing factor, proline- and glutamine-rich	*Sfpq*	75.39
45	P62264	40S ribosomal protein S14	*Rps14*	16.26
46	P62301	40S ribosomal protein S13	*Rps13*	17.21
47	O88569	Heterogeneous nuclear ribonucleoproteins A2/B1	*Hnrnpa2b1*	37.38
48	P63101	14-3-3 protein zeta/delta	*Ywhaz*	27.75
49	P62806	Histone H4	*H4c1*	11.36
50	Q9D8E6	60S ribosomal protein L4	*Rpl4*	47.12
51	P11499	Heat shock protein HSP 90-beta	*Hsp90ab1*	83.23
52	P61161	Actin-related protein 2	*Actr2*	44.73
53	P09541	Myosin light chain 4	*Myl4*	21.15
54	P84089	Enhancer of rudimentary homolog	*Erh*	12.25
55	P47754	F-actin-capping protein subunit alpha-2	*Capza2*	32.95
56	P61358	60S ribosomal protein L27	*Rpl27*	15.79
57	P62751	60S ribosomal protein L23a	*Rpl23a*	17.68
58	P19253	60S ribosomal protein L13a	*Rpl13a*	23.45
59	O08583	THO complex subunit 4	*Alyref*	26.92
60	P60867	40S ribosomal protein S20	*Rps20*	13.36
61	P53026	60S ribosomal protein L10a	*Rpl10a*	24.90
62	P62274	40S ribosomal protein S29	*Rps29*	6.67
63	P62245	40S ribosomal protein S15a	*Rps15a*	14.83
64	P02088	Hemoglobin subunit beta-1	*Hbb-b1*	15.83
65	Q9CXW4	60S ribosomal protein L11	*Rpl11*	20.24
66	P46735	Unconventional myosin-Ib	*Myo1b*	128.48
67	Q8BG05	Heterogeneous nuclear ribonucleoprotein A3	*Hnrnpa3*	39.63

Table 2. Overlapped interacted proteins for IFITM1 and IFITM3 (continued)

	Accession	Protein names	Gene names	MW [kDa]
68	Q7TPW1	Nexilin	*Nexn*	72.06
69	O55142	60S ribosomal protein L35a	*Rpl35a*	12.55
70	P14131	40S ribosomal protein S16	*Rps16*	16.44
71	P14869	60S acidic ribosomal protein P0	*Rplp0*	34.19
72	P61514	60S ribosomal protein L37a	*Rpl37a*	10.27
73	Q61495	Desmoglein-1-alpha	*Dsg1a*	114.52
74	Q9WV32	Actin-related protein 2/3 complex subunit 1B	*Arpc1b*	41.04
75	Q9CVB6	Actin-related protein 2/3 complex subunit 2	*Arpc2*	34.34
76	Q8VEK3	Heterogeneous nuclear ribonucleoprotein U	*Hnrnpu*	87.86
77	Q9QXA5	U6 snRNA-associated Sm-like protein LSm4	*Lsm4*	15.07
78	P27048	Small nuclear ribonucleoprotein-associated protein B	*Snrpb*	23.64
79	P61255	60S ribosomal protein L26	*Rpl26*	17.25
80	P47911	60S ribosomal protein L6	*Rpl6*	33.49
81	P38647	Stress-70 protein, mitochondrial	*Hspa9*	73.42
82	E9Q3S4	Mitogen-activated protein kinase kinase kinase 19	*Map3k19*	146.32
83	P62281	40S ribosomal protein S11	*Rps11*	18.42
84	O88477	Insulin-like growth factor 2 mRNA-binding protein 1	*Igf2bp1*	63.41

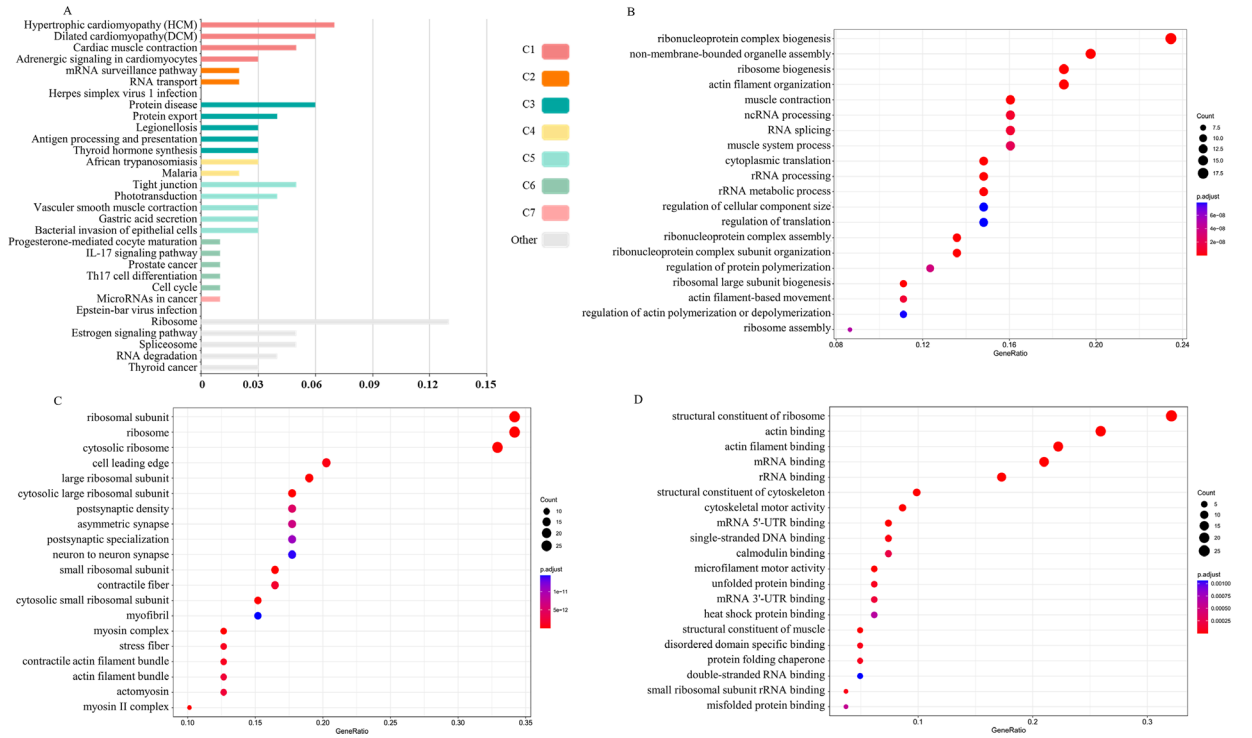


Figure 5. GO and KEGG pathway enrichment analysis of 84 proteins that interact with IFITM1, 3 (overlapped). (A) KEGG classification map of differentially expressed genes. The y-axis shows the metabolic pathway. (B) Biological process (BP). (C) Cellular component (CC). (D) Molecular function (MF). The x-axis represents gene ratio = count/set size. Dot size represents the number of genes, and the color bar represents the P_{adj}-value.

mechanisms of IFITMs, we hypothesize that cytoskeletal proteins desmin, actin, myosin, vimentin, and nestin may function as scaffolds to enroll IFITMs, thereby interacting with IFITM1 and IFITM3. Desmin regulates myoblast differentiation through its downstream myogenic determination factors MyoD, Mrf4, and myogenin, which serve as differentiation factors. Our results provide insight into the molecular mechanisms of Ifitm-mediated myogenic differentiation and may facilitate the development of future treatments for myotrophic diseases.

Funding: This work was supported by a grant from Academic Promotion Program of Shandong First Medical University (LJ001).

Conflict of Interest: The authors have no conflicts of interest to disclose.

References

1. Zhao X, Li J, Winkler CA, An P, Guo JT. IFITM Genes, Variants, and Their Roles in the Control and Pathogenesis

of Viral Infections. Front Microbiol. 2018; 9:3228.
2. Liao Y, Goraya MU, Yuan X, Zhang B, Chiu SH, Chen JL. Functional Involvement of Interferon-Inducible Transmembrane Proteins in Antiviral Immunity. Front Microbiol. 2019; 10:1097.
3. Lewin AR, Reid LE, McMahon M, Stark GR, Kerr IM. Molecular analysis of a human interferon-inducible gene family. Eur J Biochem. 1991; 199:417-423.
4. Bedford JG, O'Keeffe M, Reading PC, Wakim LM. Rapid interferon independent expression of IFITM3 following T cell activation protects cells from influenza virus infection. PLoS One. 2019; 14:e0210132.
5. Wakim LM, Gupta N, Mintern JD, Villadangos JA. Enhanced survival of lung tissue-resident memory $CD8^+$ T cells during infection with influenza virus due to selective expression of IFITM3. Nat Immunol. 2013; 14:238-245.
6. Amet T, Son YM, Jiang L, Cheon IS, Huang S, Gupta SK, Dent AL, Montaner LJ, Yu Q, Sun J. BCL6 represses antiviral resistance in follicular T helper cells. Journal of leukocyte biology. 2017; 102:527-536.
7. Yánez DC, Sahni H, Ross S, *et al*. IFITM proteins drive type 2 T helper cell differentiation and exacerbate allergic airway inflammation. European journal of immunology. 2019; 49:66-78.
8. Kim YC, Won SY, Jeong BH. The first association study of single-nucleotide polymorphisms (SNPs) of the IFITM1 gene with influenza H1N1 2009 pandemic virus infection. Molecular & cellular toxicology. 2021; 17:179-186.
9. Prelli Bozzo C, Nchioua R, Volcic M, *et al*. IFITM proteins promote SARS-CoV-2 infection and are targets for virus inhibition *in vitro*. Nature communications. 2021; 12:4584.
10. Reid LE, Brasnett AH, Gilbert CS, Porter AC, Gewert DR, Stark GR, Kerr IM. A single DNA response element can confer inducibility by both alpha- and gamma-interferons. Proceedings of the National Academy of Sciences of the United States of America. 1989; 86:840-844.
11. Lu J, Pan Q, Rong L, He W, Liu SL, Liang C. The IFITM proteins inhibit HIV-1 infection. J Virol. 2011; 85:2126-2137.
12. Savidis G, Perreira JM, Portmann JM, Meraner P, Guo Z, Green S, Brass AL. The IFITMs Inhibit Zika Virus Replication. Cell Rep. 2016; 15:2323-2330.
13. Raychoudhuri A, Shrivastava S, Steele R, Kim H, Ray R, Ray RB. ISG56 and IFITM1 proteins inhibit hepatitis C virus replication. J Virol. 2011; 85:12881-12889.
14. Tanaka SS, Matsui Y. Developmentally regulated expression of mil-1 and mil-2, mouse interferon-induced transmembrane protein like genes, during formation and differentiation of primordial germ cells. Gene Expr Patterns. 2002; 2:297-303.
15. Lange UC, Saitou M, Western PS, Barton SC, Surani MA. The fragilis interferon-inducible gene family of transmembrane proteins is associated with germ cell specification in mice. BMC Dev Biol. 2003; 3:1.
16. Buchrieser J, Degrelle SA, Couderc T, *et al*. IFITM proteins inhibit placental syncytiotrophoblast formation and promote fetal demise Interferon-induced transmembrane proteins inhibit cell fusion mediated by trophoblast syncytins. Science. 2019; 365:176-180.
17. Zani A, Zhang L, McMichael TM, Kenney AD, Chemudupati M, Kwiek JJ, Liu SL, Yount JS. Interferon-induced transmembrane proteins inhibit cell fusion mediated by trophoblast syncytins. J Biol Chem. 2019; 294:19844-19851.
18. Lange UC, Adams DJ, Lee C, Barton S, Schneider R, Bradley A, Surani MA. Normal germ line establishment in mice carrying a deletion of the Ifitm/Fragilis gene family cluster. Molecular and cellular biology. 2008; 28:4688-4696.
19. Hanagata N. IFITM5 mutations and osteogenesis imperfecta. Journal of bone and mineral metabolism. 2016; 34:123-131.
20. Hedjazi G, Guterman-Ram G, Blouin S, Schemenz V, Wagermaier W, Fratzl P, Hartmann MA, Zwerina J, Fratzl-Zelman N, Marini JC. Alterations of bone material properties in growing Ifitm5/BRIL p.S42 knock-in mice, a new model for atypical type VI osteogenesis imperfecta. Bone. 2022; 162:116451.
21. Tyurin A, Merkuryeva E, Zaripova A, Markova T, Nagornova T, Dantsev I, Nadyrshina D, Zakharova E, Khusainova R. Does the c.-14C>T Mutation in the IFITM5 Gene Provide Identical Phenotypes for Osteogenesis Imperfecta Type V? Data from Russia and a Literature Review. Biomedicines. 2022; 10.
22. Shapiro JR, Lietman C, Grover M, *et al*. Phenotypic variability of osteogenesis imperfecta type V caused by an IFITM5 mutation. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 2013; 28:1523-1530.
23. Wang D, Wang D, Huang M, *et al*. Transcriptomic characteristics and impaired immune function of patients who retest positive for SARS-CoV-2 RNA. J Mol Cell Biol. 2021; 13:748-759.
24. Kelemen A, Carmi I, Oszvald Á, Lőrincz P, Petővári G, Tölgyes T, Dede K, Bursics A, Buzás EI, Wiener Z. IFITM1 expression determines extracellular vesicle uptake in colorectal cancer. Cell Mol Life Sci. 2021; 78:7009-7024.
25. Gómez-Herranz M, Faktor J, Yébenes Mayordomo M, Pilch M, Nekulova M, Hernychova L, Ball KL, Vojtesek B, Hupp TR, Kote S. Emergent Role of IFITM1/3 towards Splicing Factor (SRSF1) and Antigen-Presenting Molecule (HLA-B) in Cervical Cancer. Biomolecules. 2022; 12.
26. Yu F, Xie D, Ng SS, Lum CT, Cai MY, Cheung WK, Kung HF, Lin G, Wang X, Lin MC. IFITM1 promotes the metastasis of human colorectal cancer *via* CAV-1. Cancer Lett. 2015; 368:135-143.
27. Yan J, Jiang Y, Lu J, Wu J, Zhang M. Inhibiting of Proliferation, Migration, and Invasion in Lung Cancer Induced by Silencing Interferon-Induced Transmembrane Protein 1 (IFITM1). Biomed Res Int. 2019; 2019:9085435.
28. Xu L, Zhou R, Yuan L, Wang S, Li X, Ma H, Zhou M, Pan C, Zhang J, Huang N, Shi M, Bin J, Liao Y, Liao W. IGF1/IGF1R/STAT3 signaling-inducible IFITM2 promotes gastric cancer growth and metastasis. Cancer Lett. 2017; 393:76-85.
29. De Marco M, Basile A, Iorio V, *et al*. Role of BAG3 in cancer progression: A therapeutic opportunity. Semin Cell Dev Biol. 2018; 78:85-92.
30. Liu X, Chen L, Fan Y, Hong Y, Yang X, Li Y, Lu J, Lv J, Pan X, Qu F, Cui X, Gao Y, Xu D. IFITM3 promotes bone metastasis of prostate cancer cells by mediating activation of the TGF-β signaling pathway. Cell Death Dis. 2019; 10:517.
31. Liu Y, Lu R, Cui W, *et al*. High IFITM3 expression predicts adverse prognosis in acute myeloid leukemia. Cancer Gene Ther. 2020; 27:38-44.
32. Liu Y, Liu J, Tian Z, Zhang Z, Liu T, Chen C, Tang X, Zhu J. Highly expressed IFITM10 is associated with early

diagnosis and T stage of gastric cancer. Transl Cancer Res. 2021; 10:382-392.
33. Agarwal M, Sharma A, Kumar P, Kumar A, Bharadwaj A, Saini M, Kardon G, Mathew SA-O. Myosin heavy chain-embryonic regulates skeletal muscle differentiation during mammalian development. LID - 10.1242/dev.184507 [doi] LID - dev184507.
34. Costa ML, Escaleira R, Cataldo A, Oliveira F, Mermelstein CS. Desmin: molecular interactions and putative functions of the muscle intermediate filament protein. Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas. 2004; 37:1819-1830.
35. Costa ML, Escaleira R, Manasfi M, de Souza LF, Mermelstein CS. Cytoskeletal and cellular adhesion proteins in zebrafish (Danio rerio) myogenesis. Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas. 2003; 36:1117-1120.
36. Zammit PS. Function of the myogenic regulatory factors Myf5, MyoD, Myogenin and MRF4 in skeletal muscle, satellite cells and regenerative myogenesis.
37. Lau SL, Yuen ML, Kou CY, Au KW, Zhou J, Tsui SK. Interferons induce the expression of IFITM1 and IFITM3 and suppress the proliferation of rat neonatal cardiomyocytes. J Cell Biochem. 2012; 113:841-847.
38. Ling SL, Zhang CW, Wang W, Cai XY, Yu L, Wu FM, Zhang LH, Tian CL. Combined approaches of EPR and NMR illustrate only one transmembrane helix in the human IFITM3. Scientific Reports. 2016; 6.
39. Zhao XS, Li JR, Winkler CA, An P, Guo JT. IFITM Genes, Variants, and Their Roles in the Control and Pathogenesis of Viral Infections. Frontiers in Microbiology. 2019; 9.
40. Akiva İ, Birgül Iyison N. Identification of IFITM3 and MGAT1 as novel interaction partners of BRI3 by yeast two-hybrid screening. Turkish journal of biology = Turk biyoloji dergisi. 2018; 42:463-470.
41. Li W, Zhang Z, Zhang L, Zhou Q, Li Y, Yi L, Ding H, Zhao M, Chen J, Fan S. Interaction of SERINC5 and IFITM1/2/3 regulates the autophagy-apoptosis-immune network under CSFV infection. Virulence. 2022; 13:1720-1740.
42. Xu Y, Yang G, Hu G. Binding of IFITM1 enhances the inhibiting effect of caveolin-1 on ERK activation. Acta Biochim Biophys Sin (Shanghai). 2009; 41:488-494.
43. Marzuca-Nassr G, Vitzel K, Mancilla-Solorza E, Márquez J. Sarcomere Structure: The Importance of Desmin Protein in Muscle Atrophy. International Journal of Morphology. 2018; 36:576-583.
44. Hnia K, Ramspacher C, Vermot J, Laporte J. Desmin in muscle and associated diseases: beyond the structural function. Cell Tissue Res. 2015; 360:591-608.
45. Sassoon DA. Myogenic regulatory factors: dissecting their role and regulation during vertebrate embryogenesis. Dev Biol. 1993; 156:11-23.
46. Weitzer G, Milner DJ, Kim JU, Bradley A, Capetanaki Y. Cytoskeletal control of myogenesis: a desmin null mutation blocks the myogenic pathway during embryonic stem cell differentiation. Dev Biol. 1995; 172:422-439.
47. Joanne P, Hovhannisyan Y, Bencze M, Daher MT, Parlakian A, Toutirais G, Gao-Li J, Lilienbaum A, Li Z, Kordeli E, Ferry A, Agbulut O. Absence of Desmin Results in Impaired Adaptive Response to Mechanical Overloading of Skeletal Muscle. Front Cell Dev Biol. 2021; 9:662133.
48. Taylor MR, Slavov D, Ku L, *et al*. Prevalence of desmin mutations in dilated cardiomyopathy. Circulation. 2007; 115:1244-1251.
49. Ojrzyńska N, Bilińska ZT, Franaszczyk M, Płoski R, Grzybowski J. Restrictive cardiomyopathy due to novel desmin gene mutation. Kardiologia polska. 2017; 75:723.
50. Walter MC, Reilich P, Huebner A, Fischer D, Schroder R, Vorgerd M, Kress W, Born C, Schoser BG, Krause KH, Klutzny U, Bulst S, Frey JR, Lochmuller H. Scapuloperoneal syndrome type Kaeser and a wide phenotypic spectrum of adult-onset, dominant myopathies are associated with the desmin mutation R350P. Brain. 2007; 130:1485-1496.
51. Capetanaki Y, Milner DJ, Weitzer G. Desmin in muscle formation and maintenance: knockouts and consequences. Cell Struct Funct. 1997; 22:103-116.
52. Babai F, Musevi-Aghdam J, Schurch W, Royal A, Gabbiani G. Coexpression of alpha-sarcomeric actin, alpha-smooth muscle actin and desmin during myogenesis in rat and mouse embryos I. Skeletal muscle. Differentiation. 1990; 44:132-142.
53. Wu Y, Zhang X, Salmon M, Lin X, Zehner ZE. TGFbeta1 regulation of vimentin gene expression during differentiation of the C2C12 skeletal myogenic cell line requires Smads, AP-1 and Sp1 family members. Biochim Biophys Acta. 2007; 1773:427-439.
54. Lindqvist J, Torvaldson E, Gullmets J, Karvonen H, Nagy A, Taimen P, Eriksson JE. Nestin contributes to skeletal muscle homeostasis and regeneration. J Cell Sci. 2017; 130:2833-2842.
55. Michalczyk K, Ziman M. Nestin structure and predicted function in cellular cytoskeletal organisation. Histol Histopathol. 2005; 20:665-671.
56. Collins K, Sellers J, Matsudaira P. Myosin I: a new insight into the mechanism and cellular significance of actin-based motility. Advances in biophysics. 1991; 27:221-226.
57. Qu G, Yan H, Strauch AR. Actin isoform utilization during differentiation and remodeling of BC3H1 myogenic cells. J Cell Biochem. 1997; 67:514-527.
58. Lee J, Robinson ME, Ma N, *et al*. IFITM3 functions as a PIP3 scaffold to amplify PI3K signalling in B cells. Nature. 2020; 588:491-497.
59. Chu PY, Huang WC, Tung SL, Tsai CY, Chen CJ, Liu YC, Lee CW, Lin YH, Lin HY, Chen CY, Yeh CT, Lin KH, Chi HC. IFITM3 promotes malignant progression, cancer stemness and chemoresistance of gastric cancer by targeting MET/AKT/FOXO3/c-MYC axis. Cell & bioscience. 2022; 12:124.
60. Min JQ, Feng Q, Liao WJ, Liang YM, Gong CW, Li EL, He WF, Yuan RF, Wu LQ. IFITM3 promotes hepatocellular carcinoma invasion and metastasis by regulating MMP9 through p38/MAPK signaling. Febs Open Bio. 2018; 8:1299-1311.

Received April 10, 2023; Revised June 15, 2023; Accepted August 15, 2023.

**Address correspondence to:*
Yanqin Lu and Jinxiang Han, Shandong First Medical University & Shandong Academy of Medical Sciences, No. 6699 Qingdao Road, Ji'nan, 250117, China.
E-mail: yqlu@sdfmu.edu.cn; jxhan@sdfmu.edu.cn

Released online in J-STAGE as advance publication August 18, 2023.

Brief Report

DOI: 10.5582/irdr.2023.01039

Analysis of microsatellite instability (MSI) in pediatric gonadal and extra-gonadal germ cell tumors

Marco Montella[1,§], Maria Elena Errico[2,§], Andrea Ronchi[1], Giuseppa Zannini[1], Vittoria Donofrio[2], Giovanni Savarese[3], Roberto Sirica[3], Francesco Esposito[4], Marco De Martino[4], Alfonso Papparella[5], Renato Franco[1], Paolo Chieffi[6,*], Federica Zito Marino[1]

[1] Pathology Unit, Department of Mental Health and Physic and Preventive Medicine, University of Campania "Luigi Vanvitelli", Naples, Italy;
[2] Pathology Unit Department of Pathology, Santobono-Pausilipon Children's Hospital, Naples, Italy;
[3] AMES, Centro Polidiagnostico Strumentale srl, Casalnuovo, Naples, Italy;
[4] Institute of Experimental Endocrinology and Oncology (IEOS) "G. Salvatore", National Research Council (CNR), Naples, Italy;
[5] Department of Child, Women, General and Specialized Surgery, University of Campania, Naples, Italy;
[6] Department of Precision Medicine, University of Campania "Luigi Vanvitelli", Naples, Italy.

SUMMARY Gonadal and extragonadal pediatric germ cell tumors (GCTs) are rare neoplasms with different clinical behavior. Although surgery and cisplatin-based chemotherapy are resolutive in most cases, some patients do not respond to chemotherapy and have a worse outcome. Microsatellite instability (MSI) was correlated to resistance to chemotherapy and sensitivity to immunotherapy in different neoplasms. A series of 21 pediatric GCTs were tested by immuno-histochemistry and PCR to evaluate MSI status. Next generation sequencing was applied to further evaluate cases with discordant results between immunohistochemistry and PCR. Twenty-one cases of pediatric GCT were included in the series. The mean age ranged between 1 and 10 years. Nine cases were gonadal GCTs and the remaining 12 were extra-gonadal GCTs. By immunohistochemistry, one case showed a deficit of Mismatch repair (MMR) proteins. This case was a 1-year-old children affected by gonadal yolk sac tumor. However, all cases resulted microsatellite stable (MSS) by PCR and NGS. MSI was not detected in our series of pediatric GCTs, as well as the data present in literature about adult patients with GCTs. Molecular techniques could have a role to confirm the MSI status in case of dMMR by immunohistochemistry.

Keywords germ cell tumor, extragonadal germ cell tumor, MSI, pediatric tumors

1. Introduction

Germ cell tumors (GCTs) are a heterogeneous group of neoplasms with different clinical behavior, occurring in gonads (gonadal germ cell tumors, GGCT) or extra-gonadal sites (extragonadal germ cell tumors, EGCT), mainly including the sacrococcygeal area, the mediastinum, and the brain (*1,2*). GCTs occurring in pediatric patients (aged 0–18 years) have some clinical and biological peculiarities, differently from the adult counter-part. Pediatric GCTs account for 3% of all malignancies and most commonly arise in children younger than 15 years, with a slight female predominance (male: female ratio: 0.8:1) (*3*). Extragonadal GCTs are relatively more frequent in pediatric patients rather than in adults. Indeed, EGCTs account for about 50% of GCTs in children and 10% of GCTs in adults. GCTs originate from the primordial multipotential germ cells that migrate along the body midline to the gonadal sites during embryogenesis. EGCTs are supposed as originating from primordial germ cells that failed to migrate to the gonads (*2,4,5*). Histologically, GCTs include undifferentiated forms (seminoma/dysgerminoma), embryonal-like differentiated forms (embryonal carcinoma and teratoma) and extra embryonal-like forms (choriocarcinoma and yolk sac tumor) (*6*).

Pediatric GCTs are an heterogenous group of neoplasms with different clinical behavior. Pure yolk sac tumor, seminoma, or mixture of the two are largely the most common histotypes in children less than 4 years of age, while tumors occurring in patients around the time of puberty up through young adulthood includes the full range of possible histotypes (*7*). Histology-specific analyses among white persons revealed that EGCTs

of the brain, pineal gland and pituitary gland were predominantly seminomas/dysgerminomas (67%, 74%, 78%, respectively). In contrast, EGCTs of the pelvis were predominately non-seminomas/non-dysgerminomas (96%) (*2*). Although the rate of malignant GCTs differs by age and location, generally about 20% of them are malignant (*7*).

Therapy of GCTs includes surgery, radiation therapy and chemotherapy. Although surgery and radiation therapy are sufficient in benign and early-stage malignant cases, cisplatin-based chemotherapy is generally applied to patients with advanced GCTs and targeted therapy currently has a limited role (*8*). Since introduction of platinum-based chemotherapy in the 1980s, survival of GCTs has dramatically improved so that the five-year survival is over than 80% (*8*). In addition, better survival has been observed for gonadal in comparison with extra gonadal tumors (*7*). Although the combination of surgery and cisplatin-based chemotherapy is resolutive in more than 90% of cases, some patients do not longer respond to chemotherapy or have a late relapse (*8*). Thus, the recognition of cases that will not benefit from conventional chemotherapy, and the use of alternative therapies, are currently needed, mainly for cisplatin-based chemotherapy-resistant patients. In this setting, microsatellite instability (MSI) may represent a promising biomarker, as it was established that different tumors carrying the deficit of Mismatch repair (MMR) proteins may present resistance to conventional chemotherapy and sensitivity to immunotherapy (*9-13*). MSI largely depends on the integrity of MMR complex, which is composed of 4 proteins (MLH1, MSH2, MSH6, and PMS2) that cooperatively detect and cut base-pair mismatches to allow correct re-synthetizations of the DNA strand (*14*). Mismatch repair (MMR) proteins are involved in DNA replication to repair errors, such as point mutations. MLH-1, PMS-2, MSH-2 and MSH-6 are the most relevant MMR proteins involved implicated in cancer development (*14*). Microsatellites are non-coding DNA regions of the hu-man genome that, like coding regions, can accumulate mutations in case of deficient MMR (dMMR). In clinical practice, the status of dMMR is detected by immunohistochemistry (IHC) to test the loss of MMR proteins, while MSI status may be directly indagated by sequencing-based methods, including PCR and next generation sequencing (NGS) (*14,15*). Particularly, the loss of one or more MMR proteins could trigger a MSI status. A proficient MMR system corrects the eventual presence of accumulated mutations, while a defective MMR system leads to global instability of both repetitive sequences and coding regions. MSI can be molecularly categorised into two distinct phenotypes: MSI-high (MSI-H) and MSI-low (MSI-L) (*14*). The former is defined as instability in two or more of the five markers in the Bethesda reference panel (BAT-25, BAT-26, D2S123, D5S346 and D17S250) is detected, while the latter is characterized by instability in only one marker (*16*). Recently, FDA approved the application of immunotherapy to any cancer with a defective MMR system and/or MSI-high genotype. The detection of MSI status has become mandatory in clinical practice for some neoplasms like colon cancer, as a strong predictor of efficacy for immunotherapy (*14*). In recent years, the role of MSI to predict the resistance to systemic chemotherapy in adult patients affected by advanced GCTs has been evaluated in some studies. Although controversial results have been reported, data seem to suggest a positive correlation between MSI and chemotherapy-resistance, and immunotherapy-sensitivity in GCTs (*14,17,18*). On the other hand, the role of MSI in pediatric GCTs is largely unknown.

In this study, we aim to assess the frequency of MSI in pediatric patients with gonadal and extragonadal GCTs, in order to identify additional molecular targets to exploit chemoresistant neoplasms or further therapeutic improvement.

2. Materials and Methods

2.1. Specimens

A series of 21 tissue samples from gonadic and extragonadal GCTs diagnosed between 2019 and 2021 at the University of Campania "L. Vanvitelli" and the "AORN Santobono-Pausilipon" Hospital were collected. Inclusion criteria were: *i*) histological diagnosis of GCT (both GGCT and EGCT) was performed; *ii*) biological material was sufficient to perform IHC and molecular tests; *iii*) age of the patient was less than 19 years at the time of the diagnosis. All 21 cases were reviewed by two experienced pathologists according to the current histological WHO classification (*6*). We retrospectively recorded clinical and pathological findings, including age of the patient at initial diagnosis, gender, tumor site and histological type.

2.2. Immunohistochemistry

Immunohistochemistry was performed on 4 μm thick whole sections for each case, using four antibodies directed against MLH1 (M1 Ventana clone ready for use and Optiview kit revelation, Tucson, AZ, USA), MSH2 (clone G219-1129 Ventana ready to use; Optiview kit revelation), MSH6 (clone SP93 Ventana ready to use; Optiview kit revelation), and PMS2 (clone A16-4 Ventana ready to use; Optiview kit revelation) proteins on the BenchMark XT device (Ventana Medical Systems). Adjacent normal tissue from each sample served as positive controls (*19, 20*). MMR protein loss was defined by the absence of IHC staining in the nucleus of tumor cells. Results were evaluated as follows: *i*) proficient MMR (pMMR), cases showing positive staining of all four MMR; *ii*) defective ex-pression of mismatch repair proteins (dMMR), cases carrying the loss of one of

two heterodimers, including MLH1/PMS2 or MSH2/MSH6 loss. We further considered another subset; *iii*) cases harboring the loss of one MMR and/or the patchy expression of one or more MMR (lopaMMR). Two independent and blinded observers carried out immunohistochemical analysis.

2.3. PCR

Serial sections of 6 μm in thickness from formalin-fixed paraffin-embedded matched normal and tumor tissues were routinely stained, and representative normal and tumor regions were identified by microscopic examination. Genomic DNA was isolated from the paraffin-embedded tissues using the QIAamp DNA mini kit (Qiagen, Valencia, CA, USA) following separation of tumor and normal tissue by manual microdissection (*21*). MSI was determined on tumor DNA using the EasyPGX® readyMSI, including the following mononucleotide repeats: BAT25, BAT26, NR21, NR22, NR24, NR27, CAT25 and MONO27. The test was performed according to the manufacturer's instructions. PCR results were evaluated as follows: *i*) microsatellite stable (MSS), cases with none of the markers unstable; *ii*) microsatellite instability-high (MSI-H), tumor with 2 or more unstable markers; *iii*) microsatellite instability-low (MSI-L), cases with only one marker unstable (in these cases new testing was carried out on non-tumor tissue, if available, to define a germinal mutation).

2.4. NGS

Tumor DNA from selected tumors was sequenced using Illumina TruSightTM Oncology 500 (TSO500) for MSI status determination. The library was prepared according to the manufacturer's protocol using a hybrid capture based TruSight Oncology 500 DNA/RNA NextSeq Kit (Illumina, San Diego, CA, USA). During library preparation, enrichment chemistry was optimized to capture nucleic acid targets from FFPE tissues. In the TSO 500 analysis, unique molecular identifiers were used to determine the unique coverage at each position and to reduce the background noise caused by sequencing and deamination artifacts in the FFPE samples. The MSI score was calculated using 130 homopolymer microsatellite loci targeted by the TSO500 panel according to the manufacturer's instructions. The proportion of unstable MSI sites to total assessed MSI sites was re-ported as a sample-level microsatellite score, in which at least 40 sites were required to determine an MSI score. The MSI status was calculated from microsatellite sites for evidence of instability relative to a set of baseline normal samples that are based on information entropy metrics.

2.5. Institutional review board statement

The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board (or Ethics Committee) of University of Campania "L.Vanvitelli" (protocol code 0007953/I, date 04/06/2020).

2.6. Informed consent statement

Our study was done retrospectively conducted on archival bio-logical samples; formal consent was not required.

3. Results and Discussion

The series analysed in our study included 21 cases of pediatric GCT. Nine out of 21 (42.9%) cases were GGCTs, and the remaining 12 (57.1%) were EGCTs. The mean age of patients was 3.6 years (range: 1–10 years). In our series, 9 out of 21 (43%) patients were male and 12 (57%) were female. Particularly, 5 out of 9 (66%) GGCTs were in testis and 4 (44%) cases in ovary. Among the 12 EGCTs, 9 cases (75%) were sacral, 2 cases (17%) were mediastinal and 1 case (8%) was coccygeal. Concerning the histology, the series included 5 Yolk sac tumor (23.8%), 11 terato-mas (52.4%) (9 mature and 2 immature), and 5 mixed GCTs (23.8%), particularly 4 yolk sac tumor and teratoma (80%) and 1 seminoma and teratoma (20%). Clinical and pathological features of the patients are summarized in Table 1.

All 21 cases were tested by IHC. Twenty cases (95%) resulted proficient MMR, while the remaining 1 case resulted dMMR (5%). The latter was a 1-year-old children affected by pure testicular yolk sac tumor. In detail, the dMMR case showed loss of expression of MLH1/PMS2 and MSH6, and low/patchy expression of MSH2, as showed in Figure 1. Clinical and pathological features and MMR status of the GCTs are detailed in Table 2. All GCT cases were adequate for PCR analysis. All 21 cases (100%) were MSS, MSS representative results of the analysis is shown in Figure 2. The NGS analysis was performed on 17 out of 21 cases, unfortunately, 4 cases of our series have not been tested since the quantity and the quality of the DNA were not adequate for this assay. All 17 cases analyzed were MSS by NGS, confirming the PCR results.

As well defined in literature, pediatric GCTs are a heterogeneous group of neoplasms, including both gonadal and extragonadal forms. Incidence of GCTs depends on the age and the sex, as these neoplasms are more frequent in adolescents and young adults (aged 15–19 years) rather than young children (age 0–4 years). In the United States the incidence rate of GCTs in children is 0.4 per 100,000 in boys and 0.6 per 100,000 in girls, while the incidence rate in adolescents and young adults is 11.4 per 100,000 in males but only one per 100,000 in females . Histologically, teratoma is largely the most common histotype in young children,

Table 1. Clinical and pathological features of the patients

Patient	Age (Year)	Sex	Site of Tumor	Histology
1	1	M	Testis (R)	Yolk Sac Tumor
2	1	M	Testis (R)	Yolk Sac Tumor
3	1	M	Mediastinum	Teratoma
4	1	M	Sacrum	Yolk Sac Tumor
5	1	F	Sacrum	Yolk Sac Tumor and Teratoma (Mature)
6	<1	F	Sacrum	Teratoma (Mature)
7	2	F	Sacrum	Yolk Sac Tumor + Teratoma (Mature)
8	1	F	Coccyx	Teratoma (Mature)
9	1	F	Sacrum	Yolk Sac Tumor
10	2	F	Sacrum	Teratoma (Mature)
11	10	F	Ovary (R)	Yolk Sac Tumor + Teratoma (Immature)
12	9	M	Testis (L)	Seminoma + Teratoma
13	<1	M	Sacrum	Teratoma
14	1	M	Testis (R)	Teratoma
15	6	F	Ovary (L)	Teratoma (Mature)
16	<1	M	Sacrum	Teratoma
17	1	M	Testis (L)	Yolk Sac Tumor
18	7	F	Ovary (R)	Teratoma (Immature)
19	8	F	Ovary (L)	Yolk Sac Tumor + Teratoma (Mature)
20	8	F	Mediastinum	Teratoma (Mature)
21	<1	F	Sacrum	Teratoma (Immature)

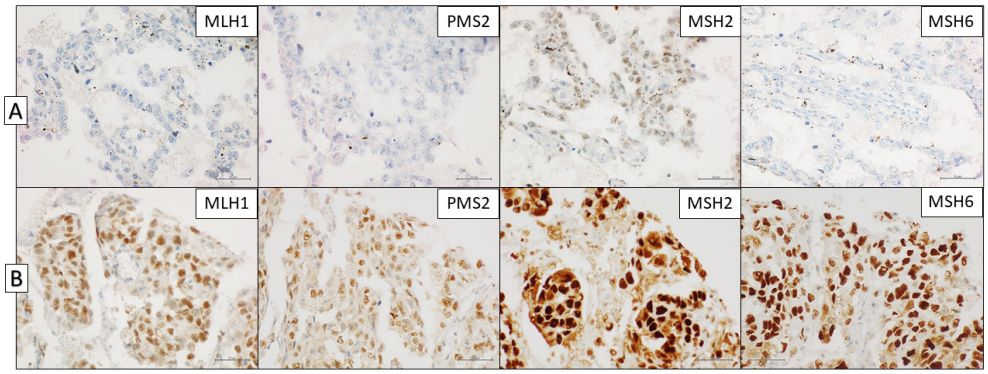


Figure 1. Immunohistochemical evaluation of MSI. (A) Loss of expression of MLH1, PMS2 and MSH6 in tumor cells with positive internal control (DAB coloration, original magnification 40x, scale bar 50μm); heterogeneous expression of MSH2 in tumor cells (DAB coloration, original magnification 40x, scale bar 50μm); **(B)** Representative case with intact expression of MLH1, PMS2, MSH2 and MSH6 (DAB coloration, original magnification 40x, scale bar 50μm).

followed by pure yolk sac tumors. In these patients, loss of chromosomes 1p, 4, 6q and gain of chromosome 1q are the most frequent cytogenetic alterations at this age, and the sacrococcygeal is the most frequent extragonadal location (*22*). In adolescents and young adults, the most common histotypes include teratoma, seminoma/dysgerminoma and mixed GCTs with a higher proportion of embryonal carcinoma and choriocarcinoma (*22*). In these patients, the most common extragonadal location is represented by the mediastinum, and isochromosome 12p (i12p) is the most common cytogenetic alteration (*22*). The clinical behavior of pediatric GCTs is variable and largely depends on histology, and sex and age of the patients. Male adolescents affected by GGCTs showed significantly worse event-free survival (EFS) than children or adults (60% *vs.* 87% and 80%, respectively) in a single-institution study (*23*). Overall, patients with advanced disease achieve a 5-year-overall survival of more than 70%, while patients with non-seminoma histology have a poorer prognosis and 5-year-overall survival of 50% (*24*). Fifteen percent of all patients will develop refractory disease, representing a challenge in the clinical management (*25*).

Although surgery may be curative in benign and early-stage malignant GCTs, advanced-stage GCTs need systemic therapy, which is mainly constituted by cisplatin-based regimens. Moreover, despite the chemo-sensitivity of GCTs, about 10-15% of cases show a resistant phenotype, responsible of tumor relapses and poor prognosis (*26*). The identification of a biomarker able to predict the chemoresistant phenotype should be important to choose the most correct therapy. In this setting, MSI could represent a promising biomarker, as it has been related to platinum-based therapy resistance and immunotherapy sensitivity in other malignant neoplasms (*14*). Two mechanisms could explain the correlation between MSI and treatment resistance. First, MSI may render cells prone to secondary mutations,

Table 2. Clinical and pathological features and MMR status of the GCTs

PATIENT	MLH1	PMS2	MSH2	MSH6	RESULTS
1	Negative	Negative	Patchy	Negative	MSI
2	Positive	Positive	Positive	Positive	MSS
3	Positive	Positive	Positive	Positive	MSS
4	Positive	Positive	Positive	Positive	MSS
5	Positive	Positive	Positive	Positive	MSS
6	Positive	Positive	Positive	Positive	MSS
7	Positive	Positive	Positive	Positive	MSS
8	Positive	Positive	Positive	Positive	MSS
9	Positive	Positive	Positive	Positive	MSS
10	Positive	Positive	Positive	Positive	MSS
11	Positive	Positive	Positive	Positive	MSS
12	Positive	Positive	Positive	Positive	MSS
13	Positive	Positive	Positive	Positive	MSS
14	Positive	Positive	Positive	Positive	MSS
15	Positive	Positive	Positive	Positive	MSS
16	Positive	Positive	Positive	Positive	MSS
17	Positive	Positive	Positive	Positive	MSS
18	Positive	Positive	Positive	Positive	MSS
19	Positive	Positive	Positive	Positive	MSS
20	Positive	Positive	Positive	Positive	MSS
21	Positive	Positive	Positive	Positive	MSS

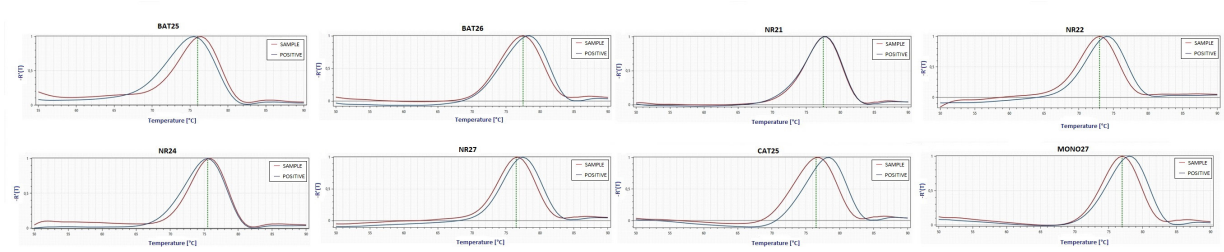


Figure 2. MSS-PCR results. Stability of BAT25, BAT26, NR21, NR22, NR24, NR27, CAT25 and MONO27 (red lines indicate samples while blue lines indicate MSS controls).

leading to resistance. Alternatively, MMR may induce S-phase cell cycle arrest and induction of apoptosis (*27*). Moreover, germ cells and GCTs are characterized by low constitutive activation of the DNA damage response machinery and respond to DNA damage with apoptosis rather than cell cycle arrest (*28*).

We performed literature research using the most diffuse database of scientific report and, to the best of our knowledge, this is the first study indagating MSI status in pediatric GCTs. MSI was indagated by immunohistochemistry, PCR and NGS, in 21 cases of pediatric (less than 10 years old patients) GCTs, including 5 Yolk sac tumor (23.8%) 11 teratomas (52.4%) and 5 mixed GCTs (23.8%). One case resulted dMMR by immunohistochemistry, showing loss of expression of MLH1/PMS2 and MSH6, and low/patchy expression of MSH2. This case was a pure yolk sac tumor occurring in the testis of a 1-year-old children. However, the MSI status was not confirmed by molecular studies, including both PCR and NGS. All cases resulted MSS by PCR. Interestingly, our series confirmed the leading role of molecular studies to define MSI status, suggesting a false-positive result of IHC (*15*).

The potential role of MSI in GCTs is largely unknow, as it was indagated in few studies, limited to adult GCTs. Devouassoux-Shisheboran *et al.* indagated the immunohistochemical expression of hMLH1 and hMSH2, showing intact nuclear staining in a series of 19 GCTs, suggesting that MMR genes may not play a significant role in early phases and progression of TGCT (*29*). In addition, previous studies showed significantly higher incidence of MSI compared with the unselected series (45 *vs.* 6%) in resistant tumours, both chemo-naïve and pre-treated cases, demonstrating that lacking expression of hMLH1 or MSH6 were significantly more frequent in resistant GCTs (*14*). Velasco *et al.* investigated the expression of the two most mutated MMR genes, MSH2 and MLH1, in sporadic testicular GCTs, founding that 25% of GCTs exhibited increased frequency of MSI. They also investigated MMR gene expression in testicular cancer as a molecular marker for clinical outcome (recurrence, response to chemotherapy and death) using protein expression and specific genetic alterations in 162 patients with testicular GCTs of different histological types (*17*). They found that tumors with altered MMR expression respond differentially to treatment, suggesting that standard platinum-based chemotherapy

does not appear to be very effective in tumors with MMR deficiency as measured by a high frequency of MSI and decreased immunostaining of MSH2 and MLH1(*17*). Al-Obaidy *et al.* analysed a series of TGCT patients including 13 patients with a subsequent contralateral TGCT. Thus, they found MLH1, PMS2, MSH2, and MSH6 retained staining in all cases of bilateral tumors (*30*).

4. Conclusion

Although further investigations on large series will be necessary to obtain more significant data, our results suggest that does not seem to be any substantial differences between adult and pediatric GCTs regarding the expression of MMRs.

Funding: None.

Conflict of Interest: The authors have no conflicts of interest to disclose.

References

1. Penn A, Jenney MEM, Nicholson JC. Germ cell tumours in children and adolescents. Paediatrics and Child Health. 2014; 24:148-154.
2. Ronchi A, Cozzolino I, Montella M, Panarese I, Zito Marino F, Rossetti S, Chieffi P, Accardo M, Facchini G, Franco R. Extragonadal germ cell tumors: Not just a matter of location. A review about clinical, molecular and pathological features. Cancer Med. 2019; 8:6832-6840.
3. Kaatsch P. Epidemiology of childhood cancer. Cancer Treat Rev. 2010; 36:277-285.
4. Chieffi P. Molecular targets for the treatment of testicular germ cell tumors. Mini Rev Med Chem. 2007; 7:755-759.
5. Chieffi P, Chieffi S, Franco R, Sinisi AA. Recent advances in the biology of germ cell tumors: Implications for the diagnosis and treatment. J Endocrinol Invest. 2012; 35:1015-1020.
6. Moch H, Cubilla AL, Humphrey PA, Reuter VE, Ulbright TM. The 2016 WHO Classification of Tumours of the Urinary System and Male Genital Organs-Part A: Renal, Penile, and Testicular Tumours. Eur Urol. 2016; 70:93-105.
7. Poynter JN, Amatruda JF, Ross JA. Trends in incidence and survival of pediatric and adolescent patients with germ cell tumors in the United States, 1975 to 2006. Cancer. 2010; 116:4882-4891.
8. Cheng L, Albers P, Berney DM, Feldman DR, Daugaard G, Gilligan T, Looijenga LHJ. Testicular cancer. Nat Rev Dis Primers. 2018; 4:29.
9. Weng J, Li S, Zhu Z, Liu Q, Zhang R, Yang Y, Li X. Exploring immunotherapy in colorectal cancer. J Hematol Oncol. 2022; 15:95.
10. Rizzo A. Immune checkpoint inhibitors and mismatch repair status in advanced endometrial cancer: Elective affinities. J Clin Med. 2022; 11:3912.
11. Luchini C, Bibeau F, Ligtenberg MJL, Singh N, Nottegar A, Bosse T, Miller R, Riaz N, Douillard JY, Andre F, Scarpa A. ESMO recommendations on microsatellite instability testing for immunotherapy in cancer, and its relationship with PD-1/PD-L1 expression and tumour mutational burden: A systematic review-based approach. Ann Oncol. 2019; 30:1232-1243.
12. De Martino M, Fusco A, Esposito F. HMGA and cancer: A review on patent literatures. Recent Pat Anticancer Drug Discov. 2019; 14:258-267.
13. De Martino M, Esposito F, Chieffi P. An update on microRNAs as potential novel therapeutic targets in testicular germ cell tumors. Intractable Rare Dis Res. 2020; 9:184-186.
14. Mayer F, Gillis AJ, Dinjens W, Oosterhuis JW, Bokemeyer C, Looijenga LH. Microsatellite instability of germ cell tumors is associated with resistance to systemic treatment. Cancer Res. 2002; 62:2758-2760.
15. Zito Marino F, Amato M, Ronchi A, *et al.* Microsatellite status detection in gastrointestinal cancers: PCR/NGS is mandatory in negative/patchy MMR immunohistochemistry. Cancers (Basel). 2022; 14:2204.
16. Vilar E, Gruber SB. Microsatellite instability in colorectal cancer-the stable evidence. Nat Rev Clin Oncol. 2010; 7:153-162.
17. Velasco A, Corvalan A, Wistuba, II, Riquelme E, Chuaqui R, Majerson A, Leach FS. Mismatch repair expression in testicular cancer predicts recurrence and survival. Int J Cancer. 2008; 122:1774-1777.
18. Masque-Soler N, Szczepanowski M, Leuschner I, Vokuhl C, Haag J, Calaminus G, Klapper W. Absence of BRAF mutation in pediatric and adolescent germ cell tumors indicate biological differences to adult tumors. Pediatr Blood Cancer. 2012; 59:732-735.
19. Pero R, Lembo F, Chieffi P, Del Pozzo G, Fedele M, Fusco A, Bruni CB, Chiariotti L. Translational regulation of a novel testis-specific RNF4 transcript. Mol Reprod Dev. 2003; 66:1-7.
20. Boscia F, Passaro C, Gigantino V, Perdona S, Franco R, Portella G, Chieffi S, Chieffi P. High levels of GPR30 protein in human testicular carcinoma in situ and seminomas correlate with low levels of estrogen receptor-beta and indicate a switch in estrogen responsiveness. J Cell Physiol. 2015; 230:1290-1297.
21. De Martino M, Esposito F, Pellecchia S, Cortez Cardoso Penha R, Botti G, Fusco A, Chieffi P. HMGA1-Regulating microRNAs Let-7a and miR-26a are Downregulated in Human Seminomas. Int J Mol Sci. 2020; 21:3014.
22. Fonseca A, Frazier AL, Shaikh F. Germ cell tumors in adolescents and young adults. J Oncol Pract. 2019; 15:433-441.
23. Cost NG, Cost CR, Geller JI, Defoor WR Jr. Adolescent urologic oncology: Current issues and future directions. Urol Oncol. 2014; 32:59-69.
24. Sirohi B, Huddart R. The management of poor-prognosis, non-seminomatous germ-cell tumours. Clin Oncol (R Coll Radiol). 2005; 17:543-552.
25. Lorch A, Rick O, Wundisch T, Hartmann JT, Bokemeyer C, Beyer J. High dose chemotherapy as salvage treatment for unresectable late relapse germ cell tumors. J Urol. 2010; 184:168-173.
26. Lobo J, Gillis AJM, van den Berg A, Looijenga LHJ. Prediction of relapse in stage I testicular germ cell tumor patients on surveillance: investigation of biomarkers. BMC Cancer. 2020; 20:728.
27. Mueller S, Schittenhelm M, Honecker F, Malenke E, Lauber K, Wesselborg S, Hartmann JT, Bokemeyer C,

Mayer F. Cell-cycle progression and response of germ cell tumors to cisplatin *in vitro*. Int J Oncol. 2006; 29:471-479.
28. Bartkova J, Rajpert-De Meyts E, Skakkebaek NE, Lukas J, Bartek J. DNA damage response in human testes and testicular germ cell tumours: biology and implications for therapy. Int J Androl. 2007; 30:282-291; discussion 291.
29. Devouassoux-Shisheboran M, Mauduit C, Bouvier R, Berger F, Bouras M, Droz JP, Benahmed M. Expression of hMLH1 and hMSH2 and assessment of microsatellite instability in testicular and mediastinal germ cell tumours. Mol Hum Reprod. 2001; 7:1099-1105.
30. Al-Obaidy KI, Trevino KE, Idrees MT. Clinicopathologic characterization of bilateral testicular germ cell tumors with immunohistochemical evaluation of mismatch repair and BRAF (V600E) genes mutations. Int J Surg Pathol. 2019; 27:619-623.

Received May 16, 2023; Revised July 27, 2023; Accepted August 15, 2023.

[§]These authors contributed equally to this work.
**Address correspondence to:*
Paolo Chieffi, Department of Precision Medicine, University of Campania "Luigi Vanvitelli", Via L. De Crecchio 80138, Naples, Italy.
E-mail: paolo.chieffi@unicampania.it (Paolo Chieffi)

Released online in J-STAGE as advance publication August 18, 2023.

Brief Report

DOI: 10.5582/irdr.2023.01051

Re-survey of 16 Japanese patients with advanced-stage hereditary motor sensory neuropathy with proximal dominant involvement (HMSN-P): Painful muscle cramps for early diagnosis

Hiroshi Shoji[1,*], Ryosuke Sakamoto[1], Chisato Saito[1], Kozo Akino[2], Masahiko Taniguchi[3]

[1] Division of Neurology, St. Mary's Hospital, Kurume, Fukuoka, Japan;
[2] A member of the House of Councilors, Tokyo Office, Japan;
[3] Department of Surgery, St. Mary's Hospital, Kurume, Fukuoka, Japan.

SUMMARY Hereditary motor and sensory neuropathy with proximal dominant involvement (HMSN-P) is an intractable neurological disease with autosomal dominant inheritance, four-limb weakness, sensory impairment, and a slowly progressive course. HMSN-P patients develop four-limb paralysis at the advanced-stage, as in amyotrophic lateral sclerosis (ALS). There is a natural 20- to 30-year course from initial painful muscle cramps and four-limb paralysis to respiratory dysfunction. A delay in the diagnosis of HMSN-P occurs due to the 20- to 30-year span from the initial symptom(s) to typical quadriplegia. Its early diagnosis is important, but the involvement of painful muscle cramps as an early symptom has not been clear. Following our earlier survey, we conducted a re-survey focusing on painful muscle cramps, assistive-device use, and hope for specific therapies in 16 Japanese patients with advanced-stage HMSN-P. Fifteen patients presented painful muscle cramps as the initial symptom, and muscle cramps in the lower abdomen including the flank were described by 10 of the patients. The presence of painful muscle cramps including those in the abdominal region may be a clue for the early diagnosis of HMSN-P. Painful abdominal cramps have not described in related diseases, *e.g.*, ALS, spinal muscular atrophy, and Charcot-Marie-Tooth disease. Recent patient-welfare improvements and advances in assistive devices including robot-suit assistive limbs are delaying the terminal state of HMSN-P. Regarding specific therapies for HMSN-P, many patients choose both nucleic acid medicine and the application of induced pluripotent stem cells as a specific therapy for HMSN-P.

Keywords painful muscle cramp, abdominal cramp, hereditary motor sensory neuropathy with proximal dominant involvement, HMSN-P, nucleic acid medicine, induced pluripotent stem cell

1. Introduction

Okinawa-type neurogenic muscular atrophy, *i.e.*, hereditary motor and sensory neuropathy with proximal dominant involvement (HMSN-P) is an intractable neurological disease that shows autosomal dominant inheritance and proximal muscle dominant-limb paralysis, and it is slowly progressive over a period of approx. 30 years (*1–3*). Advanced-stage HMSN-P results in severe amyotrophic lateral sclerosis (ALS)-like quadriplegia. HMSN-P has recently spread not only in Japan's Okinawa and Shiga prefectures but around the world, including sporadic cases in India (*4–6*). In 2017, Fujisaki *et al*. analysed the natural history of 97 patients with HMSN-P in Okinawa whose cases had been documented since 1980, and they reported a natural course of 20 to 30 years from the initial painful muscle cramps and four-limb paralysis to respiratory dysfunction (*3*).

In 2020, we used a survey to investigate whether there is a difference in the disease name notification and acceptance of HMSN-P compared to ALS (*7*). The early diagnosis of HMSN-P is important, but details such as the site and the triggers of painful muscle cramps, which is an early symptom, have not been fully analysed. Following our earlier survey (*7*), we provided the present study's re-survey to 16 patients with advanced-stage HMSN-P regarding mainly the presence of painful muscle cramps, the patients' use of assistive devices including a robot-suit hybrid assistive limb (HAL), and their hope for specific therapy for their HMSN-P.

2. Patients and Methods

In April 2022–March 2023, 16 patients with advanced-stage HMSN-P in their 50–70s in Okinawa, Japan were enrolled in this study with the cooperation of the local Patient Association (*Nozomi no Kai*). The re-survey's 10 items included the patient's age at the onset of HMSN-P, painful muscle cramps, current major symptoms, assistive-device use, and hope for specific therapy. The re-survey (see the Supplementary Materials, *http://www.irdrjournal.com/action/getSupplementalData.php?ID=153*) was approved by our Hospital's Ethics Committee (Gaku 22-1015).

The 16 patients' conditions corresponded to the advanced-stage of HMSN-P before the appearance of respiratory dysfunction described in the natural history of HMSN-P by Fujisaki *et al*. (*3*). The pairs of Patients 1 and 2, Patients 3 and 5, Patients 4 and 6, and Patients 7 and 8, and Patient 10 and 12 respectively had the same pedigree in which one parent had HMSN-P, and the remaining Patients 9, 14, and 15 each had one parent with HMSN-P. The result of genetic testing for TRK-fused gene (TFG) mutation (*8*) was positive in all 16 patients. The disease-onset age was defined as the patient's age at the onset of limb muscle weakness, and the onset age of muscle cramps similarly defined.

An upper-limb single-joint robot suit HAL had been used by six of the patients for ~3 years since 2017. As part of the regimen for the patient's familiarization with the HAL, both elbows were alternately flexed and extended for 5 min, and after a break, two similar sessions were performed as one set on 3 days; improvements were observed in the patients' hand-grip strength and finger-pinch forces (*9*).

The re-survey was sent to the Patient Association in Okinawa, which delivered the re-survey to the 16 patients for completion; the completed re-surveys were collected by the Patient Association. Ambiguous points in the patients' responses to the re-survey were resolved by a telephone inquiry, with some exceptions.

3. Results and Discussion

The results of the re-survey are summarized in Table 1. The average age of the 16 patients (9 males and 7 females) was 62.2±5.7 (SD) years. All of the patients reported experiencing painful muscle cramps; the clinical characteristics are described in Table 2. The muscle cramps began at the average age 34 years in lower legs, often ascending to the thigh and abdomen, and recurring intermittently for 10–20 years. In 10 patients, painful muscle cramps preceded limb weakness, and five patients had painful muscle cramps simultaneously with the onset of weakness of limbs. Conversely, Patient

Table 1. Re-survey of 16 Japanese patients with advanced-stage hereditary motor and sensory neuropathy with proximal dominant involvement (April 2022–March 2023)

Patient No.	Age/Sex Educ.	Genetic testing onset age+	Onset age of painful muscle cramps	Current major symptoms, use of aids, HAL use	Job, yrs	Hoped for therapy 1: Nucleic acid Medicine 2: iPS
1	66/m Univ.	35	20, lower-leg, thigh, abdomen	Prox. dominant quadriplegia* dyspnea, elec. wheelchair	40 continuing	1,2
2	72/m HS	40	20, calf cramps, ascending abdomen	Prox. dominant quadriplegia* elec. wheelchair	25	1,2 HAL no response
3	66/f HS	50	45, calf cramps, thigh, abdomen	Prox. dominant quadriplegia* dysphagia, elec. wheelchair	20	Early development 1,2
4	67/f HS	30	28, lower-leg, ascending abdomen	Muscle cramps, quadriplegia* elec. wheelchair	20	Specific therapy 1,2
5	73/m HS	65	15, lower-leg, thigh, abdomen	Quadriplegia*, cane, walker	35	Unknown
6	64/f HS	43	40, calf cramps	Quadriplegia*, cane, wheelchair, walker	10	2
7	66/m HS	50	10, lower-leg, abdomen(flank)	Quadriplegia, dysphagia, elec. wheelchair	40 continuing	1,2 HAL no response
8	72/f HS	50	40, calf cramps, abdomen	Dysphagia, quadriplegia wheelchair, death at 72 yrs	10	Not clear
9	53/m HS	35	30, lower-leg, ascending abdomen	Upper-limb paralysis, → lower-limb paralysis	30 continuing	2 HAL no response
10	60/f HS	43	43, calf cramps, finger	Muscle cramps, quadriplegia, cane, wheelchair	20	Early development 1,2
11	57/f HS	40	40, calf cramps, abdomen, neck	Muscle cramps, quadriplegia, cane, wheelchair	20	Specific therapy 1,2
12	50/m HS	38	38, calf cramps	Dyspnea, quadriplegia, cane, elec. wheelchair	24	1
13	53/m HS	30	30, calf cramps	Lower-limb paralysis	20 continuing	Not clear
14	57/f HS	40	40, lower-leg	Quadriplegia, cane, wheelchair	14	Specific therapy 2
15	60/m HS	40	50,lower-leg	Quadriplegia, cane, → wheelchair	25	Specific therapy 2
16	59/m Univ.	38	34, calf cramps, abdomen	Quadriplegia, cane, → wheelchair	30	Early development 2

+: The genetic testing and onset age are stated in the text. Educ.: education, elec: electric, f: female, HS: high school graduate, HAL: hybrid assistive limb, *: HAL use, iPS: induced pluripotent stem cell, m: male, Univ.: university graduate.

Table 2. Clinical characteristics of the HMNS-P patients with painful muscle cramps (*n*=16)

Age, yrs; mean ± SD, gender	62.2 ± 5.7 yrs 9 males, 7 females
Onset age of limb weakness, yrs; mean ± SD (range)	41.7 ± 6.1 (30-65)
Onset age of painful muscle cramps, yrs; mean ± SD (range)	32.7 ± 8.9 (10-50)
Sites:	
Lower-leg, *i.e.*, calf cramps	5 (31.3%)
Lower-leg, thigh, ascending to painful abdominal cramps	9 (56.3%)
Calf cramp, abdomen with neck	1 (6.3%)
Calf cramps, finger	1 (6.3%)
Triggers:	
Falling asleep, sleeping	5 (31.3%)
Changes in position	5 (31.3%)
Fatigue	1 (6.3%)
Sneezing	1 (6.3%)
Unknown	4 (25%)

15's painful muscle cramps started after weakness of the extremities.

Two patients described neck or finger cramps together with lower-leg or abdominal cramps. The triggers included fatigue, falling asleep during the day or at night, changes in posture, and sneezing. These were relieved by stretching the lower legs, lightly tapping the abdomen, or standing up. In a few patients in their 50–60s, occasional muscle cramps still occur. Regarding medications for HMSN-P, some of the patients had received clonazepam or taurine (data not shown).

Fifteen patients described painful muscle cramps as the initial symptom, and muscle cramps in the lower abdomen, including the flank were described by 10 of the patients. Notably, painful abdominal cramps seemed to be specific to HMSN-P. Painful abdominal cramps have not described in related diseases, *e.g.*, ALS, spinal bulbar muscular atrophy (SBMA), or Charcot-Marie-Tooth (CMT) disease (*10–13*). Fasciculation in the face during tongue thrust has been observed in ALS and SBMA patients but not HMSN-P, and CMT disease shows findings of lower-extremity predominance.

Takashima *et al*. reported that muscle cramps occurred intermittently in the extremities and abdominal regions of their series of 23 patients with HMSN-P, and there was an interval ranging from 0 to 22 years between the muscle cramps and four-limb weakness (*1*). In our series of 16 patients, the corresponding interval ranged from 0 to 50 years; 10 patients had prodromal signs 3–50 years before limb weakness, and five patients had coexisting limb weakness with the onset of muscle weakness. In both types of patients, the presence of painful abdominal cramps in the present history may be a clue for the early diagnosis of HMSN-P.

Regarding the pathology of HMSN-P, Suehara described the loss of anterior horn cells throughout the spinal cord, marked atrophy of the dorsal column, and a loss of nerve fibers (*2*). The pathophysiology of abdominal muscle cramps appears to originate in the lumbar spinal anterior horn. On the other hand, muscle weakness in the upper extremities is always observed in HMSN-P, and it is presumed that muscle cramps frequently occur in the upper extremities of HMSN-P patients; however, only two patients in our present series described experiencing muscle cramps in the neck or fingers.

In six of our patients in their 60s, their current main symptoms were proximal dominant quadriplegia, and they needed an electric wheelchair and full assistance. However, they had maintained their cognitive ability, speech, and swallowing, and the painful muscle cramps were reduced in three of the six patients. They had an average of 24 years of work experience, and their jobs varied from company representative, nurse, sales worker, US military base driver, and clerical workers. Almost all of the patients had been obliged to stop working due to progressive limb weakness, but Patients 1, 7, 9, and 13 had maintained sufficient hand muscle strength to continue clerical work.

Compared to the responses to the same survey completed by the patients 2 years earlier, Patient 1 had developed dyspnea, Patient 3 had mild dysphagia, and Patient 6 was hospitalized for a hip fracture. Patient 8 had died of aspiration pneumonia. Patient 12's condition had progressed to quadriplegia, and he needed an electric wheelchair.

The present study's re-survey revealed that an upper-limb single-joint robot suit HAL was used intermittently by six patients for 3 years; Patient 1 experienced an immediate effect for 2–3 weeks in the evaluation items of hand-grip and finger-pinch forces at distal upper-limb muscles (*9*). Regarding the re-survey questions about the usefulness of an upper-limb single-joint robot suit HAL, two patients with mild disability and one patient with severe disability described the HAL as helpful; two patients with severe disability chose to maintain the HAL, and one patient with severe disability answered 'no' to the question about the HAL's helpfulness.

Comparing the patient group of 65–70 years old reported by Fujisaki *et al.* (*3*) with the seven present patients of the same age, we observed that > 50% of the patients in the Fujisaki series were using a respirator or had died, but six of our patients in that age group remained without respirator use, which shifted the terminal stage by several years. The improvement in the patients' welfare and the progress that has been made in the design of assistive devices are considered to have greatly contributed to a delay in the onset of terminal-stage HMSN-P. The hoped-for therapy in the survey we provided 2 years ago (*7*) resulted in the same number of specific therapies desired, *i.e.*, nucleic acid medicine and induced pluripotent stem cell (iPS) application. In the present study's re-survey, one patient chose nucleic acid medicine, and five patients selected iPS application. Seven patients chose both options, and

three patients' responses were unclear or not given.

A limitation of this study is that it enrolled only patients with advanced-stage HMSN-P who belonged to the Okinawa Patient Association. In addition, all of the patients are Japanese, and our findings may not be applicable to patients with other backgrounds. A wide range of investigations, including patients from outside Okinawa prefecture, are awaited.

In conclusion, we conducted a re-survey for 16 Japanese patients with advanced-stage HMSN-P concerning mainly painful muscle cramps as an early symptom of this disease. Of particular note, the presence of painful muscle cramps including the abdominal region may be a clue for the early diagnosis of HMSN-P. Recent improvements in patients' welfare and advances in assistive devices including robot-suit assistive HALs have postponed the terminal state of HMSN-P by several years. Many of our patients chose both nucleic acid medicine and iPS application as their hoped-for HMSN-P therapy.

Acknowledgements

We sincerely thank Mr. Seiken Ganeko, the *Nozomi no Kai* representative, for his cooperation and assistance.

Funding: This study was supported by a Health Labour and Welfare Science Research Grant (no. 21FC1006) and by the Research Program for Conquering Intractable Diseases from the Japan Agency for Medical Research and Development (AMED) (no. JP22ek0109616h).

Conflict of Interest: The authors have no conflicts of interest to disclose.

References

1. Takashima H, Nakagawa M, Nakahara K, Matsuzaki T, Higuchi I, Higa H, Arimura K, Iwamasa T, Izumo S, Osame M. A new type of hereditary motor and sensory neuropathy linked to chromosome. Ann Neurol. 1997; 41:771-780.
2. Suehara M. Shinkei network series. Hereditary motor and sensory neuropathy with proximal dominant involvement. Iryo. 2001; 55:101-103. (In Japanese)
3. Fujisaki N, Suwazono S, Suehara M, Nakachi R, Kido M, Fujiwara Y, Oshiro S, Tokashiki T, Takashima H, Nakagawa M. The natural history of hereditary motor and sensory neuropathy with proximal dominant involvement (HMSN-P) in 97 Japanese patients. Intractable Rare Dis Res. 2018; 7:7-12.
4. Nakagawa M. A wide spectrum of hereditary motor sensory neuropathy (HMSN). Rinshou Shinkeigaku. 2009; 49:950-952. (in Japanese)
5. Mori C, Saito T, Saito T, Fujimura H, Sakoda S. Two patients of hereditary motor and sensory neuropathy with proximal dominant involvement (HMSN-P). Rinsho Shinkeigaku. 2015; 55:401-405. (in Japanese)
6. Ansari AF, Jagiasi K, Ojha P, Ansari R, Nagendra S, Kharat. S, Dominant P. Hereditary motor and sensory neuropathy with TFG mutation: First patient report from India. Neurol India. 2022; 70:1220-1222.
7. Taniguchi M, Yorishima Y, Shoji H, Ide M, Kumura Y, Kunisaki K. Survey of patients with advanced-stage Okinawa-type neurogenic muscular atrophy (hereditary motor and sensory neuropathy with proximal dominant involvement: HMSN-P). Rinshou Shinkeigaku. 2022; 62:152-156. (in Japanese)
8. Ishiura H, Sako W, Yoshida M, *et al*. The TRK-fused gene is mutated in hereditary motor and sensory neuropathy with proximal dominant involvement. Am J Hum Genet. 2012; 91:320-329.
9. Taniguchi M, Shoji H, Ide M, Kumura Y, Kunisaki K. Usefulness of upper-limb, single-joint-type hybrid assistive limb (HAL) for Okinawa-type neurogenic muscular atrophy (HMSN-P). A patient report. Neurology. 2021; 94:551-555. (in Japanese)
10. Liu J, Yi Li, Niu J, Zhang L, Fan J, Guan Y, Cui L Liu M. Fasciculation differences between ALS and non-ALS patients: An ultrasound study. BMC Neurol. 2021; 21:441-448.
11. Yamada S, Hirunagi T, Sahashi K, Katsuno M. Spinal bulbar muscular atrophy and spinal muscular atrophy. Nichinaikaishi. 2023; 111:1532-1540. (in Japanese)
12. Gwak DW, Jung SH, Min YS, Park JS, Cho HJ, Park D, Hong MW, Kang MG. Correlation between maximal tongue pressure and swallowing function in spinal and bulbar muscular atrophy. Front Neurol. 2021; 12:704788.
13. Morena J, Gupta A, Hoyle JC. Charcot-Marie-Tooth: From molecules to therapy. Int J Mol Sci. 2019; 20:3419.

Received June 25, 2023; Revised August 7, 2023; Accepted August 15, 2023.

**Address correspondence to:*
Hiroshi Shoji, Division of Neurology, St. Mary's Hospital, 422 Tsubukuhonmachi, Kurume, Fukuoka 830-8543, Japan.
E-mail: hshoji@st-mary-med.or.jp

Released online in J-STAGE as advance publication August 18, 2023.

Correspondence

DOI: 10.5582/irdr.2023.01033

Carbonic anhydrase II deficiency syndrome with amelogenesis imperfecta linked to a homozygous *CA2* deletion

Luan Deives Rodrigues Leite[1], Kêmelly Karolliny Moreira Resende[1], Lídia dos Santos Rosa[2], Juliana Forte Mazzeu[3], Livia Claudio de Oliveira[4], Maria do Carmo Sorci Dias Scher[4], Ana Carolina Acevedo[2], Paulo Marcio Yamaguti[1,*]

[1] Oral Care Center for Inherited Diseases, Unit of Oral Health, University Hospital of Brasilia, University of Brasilia, Brasilia, Brazil;
[2] Laboratory of Oral Histopathology, Department of Dentistry, Faculty of Health Sciences, University of Brasilia, Brasilia, Brazil;
[3] Laboratory of Clinical Genetics, Faculty of Medicine, University of Brasilia, Brasilia, Brazil;
[4] Unit of Pediatric Nephrology, University Hospital of Brasilia, University of Brasilia, Brasilia, Brazil.

SUMMARY We performed a study to present a phenotypic and genotypic characterization of a patient clinically diagnosed with carbonic anhydrase II (CAII) deficiency syndrome. Medical records were reviewed, and oral examination was performed. Sanger sequencing was undertaken for molecular diagnosis. The patient presented with osteopetrosis, renal tubular acidosis, cerebral calcification, blindness, deafness, and development delay. The oral manifestations included anterior open bite, posterior crossbite, tooth eruption impairment, and hypoplastic amelogenesis imperfecta (AI). Molecular analysis revealed a *CA2* homozygous deletion (c.753delG, p.Asn252Thrfs*14) and confirmed the clinical diagnosis. This study suggests that AI can be another feature of CAII deficiency syndrome. For the first time, a *CA2* disease-causing variant is reported to be associated with syndromic AI.

Keywords amelogenesis imperfecta, osteopetrosis, renal tubular acidosis, carbonic anhydrase II

Carbonic anhydrase II (CAII) deficiency syndrome (#OMIM 259730), also known as marble brain disease or Guibaud-Vainsel syndrome, is an autosomal recessive disease characterized by an increased bone density and metabolic acidosis that cause osteopetrosis, renal tubular acidosis and cerebral calcification (*1*). Affected individuals usually present with cranial nerve compression causing intellectual disability, blindness and deafness, recurrent bone fractures, and nephrocalcinosis. CAII is a cytoplasmic enzyme encoded by the *CA2* gene, mainly expressed in bone, brain, distal renal tubules, and erythrocytes, regulating intracellular pH by catalyzing the conversion of carbonic acid (H_2CO_3) to bicarbonate (HCO_3-) and hydrogen ion (H^+). CAII is involved in osteoclast differentiation, bone resorption, and in the acid-base physiology of the kidneys (*2*).

In recent years, experimental studies in mice have also demonstrated that ameloblasts express CAII during amelogenesis, especially in the transition and maturation stage (*3-5*). In mice, this protein plays a role in intracellular pH control, which is essential for normal enamel development. However, the enamel phenotype description has not been the focus of the clinical reports. Tooth eruption disturbances, severe dental caries, crowded teeth, and dental malocclusion are the most common oral manifestations reported (*6-8*).

Amelogenesis imperfecta (AI) comprises a heterogeneous group of inherited development defects of enamel (DDE) (*9*). AI can be an isolated or syndromic trait that affects all or almost all teeth in both dentitions. The mode of inheritance can be autosomal recessive, autosomal dominant, or X-linked. In hypoplastic AI, the enamel thickness or shape is altered; in hypomaturation and hypocalcified AI, the enamel matrix mineralization is incomplete, and enamel hardness is reduced.

The present study aimed to perform a phenotypic and genotypic characterization in a current 26-year-old female Brazilian patient (III:1) clinically diagnosed with CAII deficiency at age nine years old.

The study was approved by the Research Ethics Committee, Faculty of Health Sciences, University of Brasilia (certificate of presentation for ethical appreciation number 43064320.3.0000.0030) in accordance with the Declaration of Helsinki. Informed consent was obtained from the participants.

The proband was the only affected member in the family (Figure 1, A). Parents reported no consanguinity, but both were born in a small village in the State of

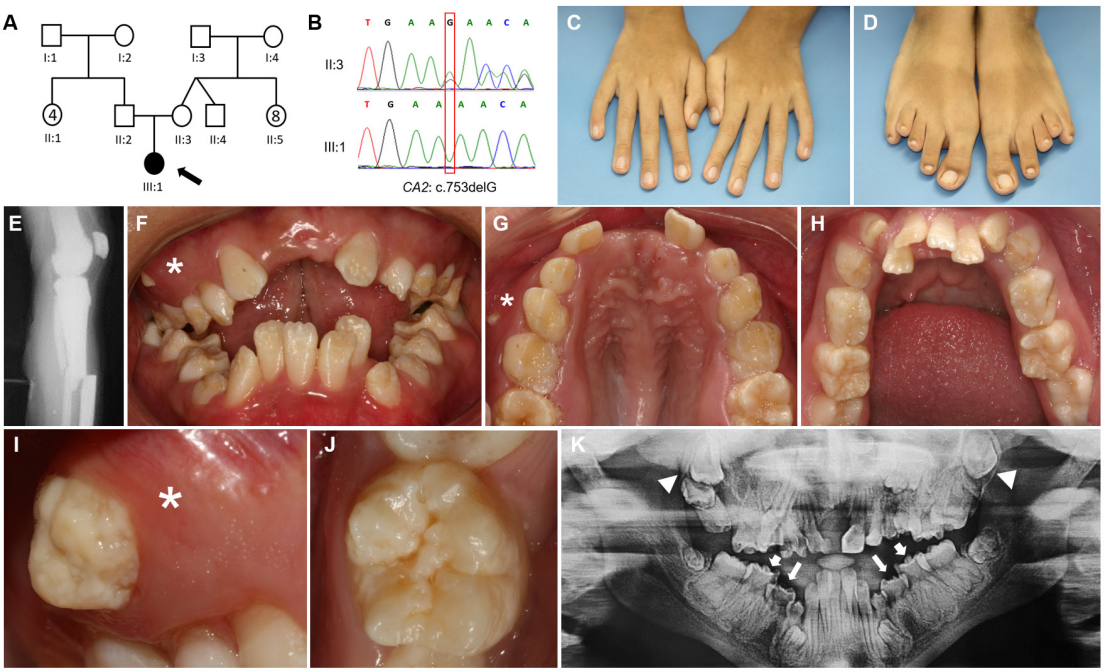


Figure 1. Genetic, clinical, and radiographic findings of this study. (A) Pedigree illustrates the absence of other affected members in the family or parental consanguinity. **(B)** Chromatograms show mother heterozygosity and proband homozygosity for the *CA2* variant. **(C)** Physical extraoral examination detected slender fingers and bilateral 5th finger clinodactyly in upper limbs. **(D)** It was also observed hammertoe bilaterally (1st toes), sandal gap, and short 3rd/4th toes in lower limbs. **(E)** Radiography of knee and leg showing tibial and fibular fractures. **(F)** Intraoral examination at age 17: anterior open bite, posterior crossbite with crowded teeth, and ectopic eruption (*). **(G)** The occlusal view shows a high-arched palate with maxillary atresia. **(H)** Crowded teeth in the mandible and primary second molars retention. **(I)** Ectopic eruption of maxillary first premolar with hypoplastic pitting enamel (*). **(J)** Approximate view of a lower permanent first molar with hypoplastic pitting enamel. **(K)** Dental panoramic radiography at age 26 revealed retention of primary molars, eruption failure of upper permanent molars (white triangles), and abnormal enamel thickness in primary and permanent molars (white arrows).

Maranhão, Brazil. She was an only child, delivered from an uncomplicated pregnancy. She presented with osteopetrosis, microcephaly, cerebral calcifications, developmental delay, short stature, limb malformations, and recurrent fractures of the femur, tibia, and fibula (Figure 1, C-E). Furthermore, she presented right optic nerve atrophy with visual impairment, strabismus, horizontal nystagmus, and conductive hearing loss. At age nine years old, she presented with low values of venous blood pH (7.21), low HCO_3 (16.7 mEq/L) and low base excess (-10.5 mmol/L); high urinary pH (6.0); high urinary excretion of calcium (5.4 mg/kg/24 h), and low excretion of citrate (0.32 mmol/24 h). She was diagnosed with distal renal tubular acidosis, metabolic acidosis, hypercalciuria, and hypocitraturia.

At age 9, she was referred to the Oral Care Center for Inherited Diseases, University Hospital of Brasilia, University of Brasilia, and a complete oral examination was performed. She presented mixed dentition with generalized enamel hypoplasia, high-arched and ogival palate, anterior open bite, posterior crossbite, crowded teeth, ectopic eruption, and altered tooth shape. Later, at age 17, she presented with retained primary and impacted permanent teeth. All primary and permanent teeth presented with hypoplastic pits and grooves resembling hypoplastic AI (Figure 1, F-J). She also presented with gingival inflammation in areas of crowded teeth due to difficult access for oral hygiene. Dental panoramic radiography showed tooth eruption impairment and impacted teeth (Figure 1, K).

Venous blood was collected from the proband and the mother for DNA isolation. *CA2* gene was amplified using specific pairs of primers (available upon request), and PCR products were sequenced. Sanger sequencing results were compared with *CA2* reference sequences (*http://www.ensembl.org*) and revealed that the proband was homozygous and the mother (II:3) was heterozygous for a single base pair deletion resulting in a frameshift mutation in exon 7 (*CA2*: c.753delG, p.Asn252Thrfs*14) (Figure 1, B). The variant was predicted to be likely pathogenic (PM2, PVS1, PP4) according to the American College of Medical Genetics and Genomics (ACMG) criteria (*10*). The father (II:2) was not available for the genetic exam. The variant nomenclature was checked on the Mutalyzer Website (*https://mutalyzer.nl*) and registered in the Leiden Open Variation Database (LOVD).

In this study, the patient presented with the classic features of CAII deficiency syndrome, except for the absence of nephrocalcinosis, and the presence of hypoplastic AI, which had never been previously reported in this syndrome. The same variant detected in our patient had already been reported in two Mexican siblings (*11*), but the orodental features were not described.

CAII deficiency cases present a particular geographic distribution and have been mostly reported in the Mediterranean region and the Middle East (*6,12*), *i.e*. patients of Arab descent, probably due to the high occurrence of consanguineous marriages in this population. In the present family, no consanguinity was reported, but once the parents are natives from a small village with a reduced population, the possibility of familial genetic similarities between them may be considered to explain the homozygosity detected in the proband.

We performed a literature search in 3 databases (Medline, PubMed, and Web of Science) until July 2023, covering the topics: "CAII deficiency OR carbonic anhydrase 2" AND "oral manifestations OR enamel". After the removal of duplicates and reports in foreign languages, a total of 309 studies were retrieved. Abstracts were screened, and 60 records were eligible for the next phase. After reading the complete studies, we identified only four case reports (*13-16*) and one series case report (*6*) that observed enamel hypoplasia in some patients. Among them, only one case report confirmed the molecular etiology and described the presence of enamel hypoplasia in three patients from two unrelated families (*13*). None of them mentioned generalized DDE resembling AI.

The renal phenotype of *Car2*(-/-) mice are similar to the observed in CAII deficiency patients (*17*), *i.e.*, metabolic acidosis and impaired urine acidification, but there is no transgenic *Ca2* animal models study with an orodental approach in the literature. Our patient presented AI in both primary and permanent dentition. As the enamel development of primary dentition occurs during prenatal life, when the placenta provides the fetal homeostasis for amelogenesis, it may be assumed that DDE in primary teeth are unlikely to be caused by systemic disturbances but by an inherited condition in patients born from uncomplicated pregnancies (*18*). DDE may also be caused by other factors such as local infections, dental trauma, environmental and systemic disturbances. Thus, a secondary effect of the metabolic acidosis found in our patient cannot be discharged once the ameloblasts are sensitive to pH changes during amelogenesis.

The CAII role during amelogenesis has only recently been described (*3-5*), and it may explain why dental enamel phenotype has not been carefully characterized in previous studies. Furthermore, in the past, AI used to be classified only as an isolated trait caused by variants in genes that encoded for enamel matrix proteins or proteases. Advances in molecular diagnosis techniques have identified disease-causing variants in at least 26 different genes that cause 19 types of syndromic AI (*9*), including five autosomal recessive renal diseases linked to *CLDN16* (#OMIM 248250), *CLDN19* (#OMIM 248190), *FAM20A* (#OMIM 204690), *KCNJ1* (#OMIM 241200) and *SLC4A4* genes (#OMIM 604278). All these genes are functionally expressed in both ameloblasts and kidney cells.

In conclusion, the generalized enamel hypoplasia observed in the primary and permanent teeth in the present case suggests that AI can be part of the phenotypic spectrum of CAII deficiency syndrome. Additionally, we suggest that this syndrome should be included in the hall of syndromic AI and, more specifically, in the group of autosomal recessive diseases with renal and dental enamel involvement. These results reinforce the importance of referring patients with autosomal recessive renal diseases for oral examination. Likewise, AI patients should be referred for nephrological evaluation.

Acknowledgements

We thank the study participants for their kind cooperation.

Funding: This study was supported by CAPES/COFECUB, National Council for Scientific and Technological Development (CNPq), and *Decanato de Pesquisa e Inovação*, University of Brasilia.

Conflict of Interest: The authors have no conflicts of interest to disclose.

References

1. Whyte MP. Carbonic anhydrase II deficiency. Bone. 2023; 169:116684.
2. Borthwick KJ, Kandemir N, Topaloglu R, Kornak U, Bakkaloglu A, Yordam N, Ozen S, Mocan H, Shah GN, Sly WS, Karet FE. A phenocopy of CAII deficiency: A novel genetic explanation for inherited infantile osteopetrosis with distal renal tubular acidosis. J Med Genet. 2003; 40:115-121.
3. Bori E, Guo J, Rácz R, Burghardt B, Földes A, Kerémi B, Harada H, Steward MC, Den Besten P, Bronckers AL, Varga G. Evidence for bicarbonate secretion by ameloblasts in a novel cellular model. J Dent Res. 2016; 95:588-596.
4. Yin K, Paine ML. Bicarbonate transport during enamel maturation. Calcif Tissue Int. 2017; 101:457-464.
5. Lin HM, Nakamura H, Noda T, Ozawa H. Localization of H^+-ATPase and carbonic anhydrase II in ameloblasts at maturation. Calcif Tissue Int. 1994; 55:38-45.
6. Awad M, Al-Ashwal AA, Sakati N, Al-Abbad AA, Bin-Abbas BS. Long-term follow up of carbonic anhydrase II deficiency syndrome. Saudi Med J. 2002; 23:25-29.
7. Pang Q, Qi X, Jiang Y, Wang O, Li M, Xing X, Dong J, Xia W. Two novel CAII mutations causing carbonic anhydrase II deficiency syndrome in two unrelated Chinese families. Metab Brain Dis. 2015; 30:989-997.
8. Alsemari A, Alsuhaibani M, Alhathlool R, Ali BM. Potential oligogenic disease of mental retardation, short stature, spastic paraparesis, and osteopetrosis. Appl Clin Genet. 2018; 11:129-134.
9. Dong J, Ruan W, Duan X. Molecular-based phenotype

variations in amelogenesis imperfecta. Oral Dis. 2023; doi: 10.1111/odi.14599
10. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, Grody WW, Hegde M, Lyon E, Spector E, Voelkerding K, Rehm HL; ACMG Laboratory Quality Assurance Committee. Standards and guidelines for the interpretation of sequence variants: A joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med. 2015; 17:405-424.
11. Hu P, Lim E, Ciccolella J, Strisciuglio P, Sly W. Seven novel mutations in carbonic anhydrase II deficiency syndrome identified by SSCP and direct sequencing analysis. Hum Mutat. 1997; 9:383-387.
12. Sly WS, Whyte MP, Sundaram V, *et al*. Carbonic anhydrase II deficiency in 12 families with the autosomal recessive syndrome of osteopetrosis with renal tubular acidosis and cerebral calcification. N Engl J Med. 1985; 313:139-145.
13. Ismail EA, Abul Saad S, Sabry MA. Nephrocalcinosis and urolithiasis in carbonic anhydrase II deficiency syndrome. Eur J Pediatr. 1997; 156:957-962.
14. Ohlsson A, Stark G, Sakati N. Marble brain disease: Recessive osteopetrosis, renal tubular acidosis and cerebral calcification in three Saudi Arabian families. Dev Med Child Neurol. 1980; 22:72-84.
15. Strisciuglio P, Sartorio R, Pecoraro C, Lotito F, Sly WS. Variable clinical presentation of carbonic anhydrase deficiency: Evidence for heterogeneity? Eur J Pediatr. 1990; 149:337-340.
16. Nagai R, Kooh SW, Balfe JW, Fenton T, Halperin ML. Renal tubular acidosis and osteopetrosis with carbonic anhydrase II deficiency: Pathogenesis of impaired acidification. Pediatr Nephrol. 1997; 11:633-636.
17. Hains DS, Chen X, Saxena V, Barr-Beare E, Flemming W, Easterling R, Becknell B, Schwartz GJ, Schwaderer AL. Carbonic anhydrase 2 deficiency leads to increased pyelonephritis susceptibility. Am J Physiol Renal Physiol. 2014; 307:F869-F880.
18. Koch MJ, Bührer R, Pioch T, Schärer K. Enamel hypoplasia of primary teeth in chronic renal failure. Pediatr Nephrol. 1999; 13:68-72.

Received April 19, 2023; Revised August 18, 2023; Accepted August 21, 2023.

**Address correspondence to:*
Paulo Marcio Yamaguti, Oral Care Center for Inherited Diseases, Unit of Oral Health, University Hospital of Brasilia, University of Brasilia, Brasilia, DF, Brazil.
E-mail: paulomarcioyamaguti@gmail.com

Released online in J-STAGE as advance publication August 23, 2023.

Letter

DOI: 10.5582/irdr.2023.01069

PIK3CA mutations in cutaneous squamous cell carcinoma

Yudo Kusaba, Ikko Kajihara*, Ryoko Sakamoto, Saki Maeda-Otsuka, Saori Yamada-Kanazawa, Soichiro Sawamura, Katsunari Makino, Jun Aoi, Shinichi Masuguchi, Satoshi Fukushima

Department of Dermatology and Plastic Surgery, Faculty of Life Sciences, Kumamoto University, Kumamoto, Japan.

SUMMARY Oncogenic *PIK3CA* mutation activates phosphoinositide 3-kinase (PI3K) enzyme, and PI3K-AKT signaling activation induces several growth-regulatory transcription factors. *PIK3CA* mutations have attracted attention as biomarker in clinical trials of various inhibitors including PI3K inhibitors. About 80% of *PIK3CA* mutations in human cancers are observed in 'hot spot' regions: exon 9 (E542K and E545K) and exon 20 (H1047R). There were few reports about clinical significance of *PIK3CA* mutations in cutaneous cell carcinoma (cSCC). Thus, we investigate the prevalence of three *PIK3CA* hot spot mutations in 143 cases with cSCC and evaluate the correlation between the presence of these mutations and clinical characteristics by using ddPCR. The frequency of each E542K, E545K and H1047R PIK3CA mutations was 1.4% (2/143), 2.8% (4/143), and 0.7% (1/143) respectively. No significant correlation was found between *PIK3CA* mutations and clinical characteristics. Although additional basic researches and clinical trials are necessary, various inhibitors may be effective therapeutics for *PIK3CA* mutation-positive cSCC. Our study revealed the prevalence of *PIK3CA* mutations in cSCC.

Keywords cutaneous squamous cell carcinoma, *PIK3CA* mutations, Droplet digital polymerase chain reaction (ddPCR), metastasis

PI3K (Phosphoinositide 3-kinase)-Akt pathway can be activated by *PIK3CA* mutations and contributes to cancer progression. *PIK3CA* mutations have been found in various cancers, such as breast and colon cancer, and approximately 80% of them are in "hot spot" regions such as exon 9 (E542K and E545K) and exon 20 (H1047R) (*1*). To our knowledge, there are only two reports of *PIK3CA* mutations in cutaneous squamous cell carcinoma (cSCC). None of the *PIK3CA* hot spot mutations were observed in 30 patients with cSCC (*2*). In addition, 40% (4/10) of cases with cSSC had *PIK3CA* mutations in regions outside of hot spots, and no *PIK3CA* hot spot mutations were identified in 10 patients with cSCC (*3*). However, there have been no reports of the *PIK3CA* mutations in a large number of SCC cases. Therefore, we investigated the prevalence and clinical significance of three *PIK3CA* hot spot mutations (E542K, E545K, and H1047R) in 143 cases with cSCC.

All experimental protocols were approved by the Institutional Review Board and were carried out in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients. Tissue samples were obtained from 143 patients diagnosed at Kumamoto University Hospital between July 2015 and August 2020. DNA was isolated from formalin-fixed paraffin-embedded FFPE tissues using the QIAmp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). ddPCR was performed using a QX200 droplet digital PCR system (Bio-Rad, Berkeley, CA, USA) as previously described4 using ddPCR probes for three PIK3CA mutations (E542K, E545K, and H1047R), which were also purchased from Bio-Rad. The variant allele frequency (VAF) cutoff was 1% as in our previous study (*4*).

The overall frequency of the *PIK3CA* hot spot mutations in cSCC was 4.9% (7/143), and the frequencies of E542K, E545K, and H1047R were 1.4% (2/143), 2.8% (4/143), and 0.7% (1/143), respectively. No case with multiple *PIK3CA* hot spot mutations was observed. No significant correlations between the *PIK3CA* mutations (total, E542K, E545K, and H1047R) and clinical characteristics were found (Table 1).

Our study revealed that the overall prevalence of *PIK3CA* hot spot mutations in cSCC was approximately 5%, which differed from past reports (*2,3*). This discrepancy may be attributable to differences in research method and patient cohorts. Recently, *PIK3CA*

Table 1. Correlations between PIK3CA mutations and clinical characteristics in cutaneous squamous cell carcinomas

Clinical characteristics	E542K			E545K			H1047R			Total		
	+ (n = 2)	- (n = 141)	P	+ (n = 4)	- (n = 139)	P	+ (n = 1)	- (n = 142)	P	+ (n = 7)	- (n = 136)	P
Age (yares : mean ± SD)	80.3 ± 10.4	83.0 ± 5.0	0.875	80.3 ± 10.5	82.3 ± 6.4	0.212	80.4 ± 10.4	69.0 ± 0	NA	80.3 ± 10.5	80.6 ± 7.3	0.785
Sex (male : female)	2:0	91:50	0.542	2:2	91:48	0.612	1:0	92:50	> 0.999	5:2	88:48	> 0.999
Lymph node metastasis (– : +)	2:0	130:11	> 0.999	4:0	128:11	> 0.999	0:1	132:10	0.077	6:1	126:10	0.436
Distant organ metastasis (– : +)	2:0	140:1	> 0.999	4:0	138:1	> 0.999	1:0	141:1	> 0.999	7:0	135:1	> 0.999

NA: not assessed, SD: standard deviation.

mutations have attracted attention as biomarker in clinical trials of various inhibitors including PI3K inhibitor (5). Taken together, various inhibitors may be effective therapeutics for *PIK3CA* mutation-positive cSCC, although additional basic researches and clinical trials are necessary to confirm this.

This study has several limitations. First, *PIK3CA* mutations other than hot spot mutations were not investigated, and second, the number of cases with lymph node metastasis and distant organ metastasis was low.

Acknowledgements

We thank Hironobu Ihn for his generous kindness and assistance over the years.

Funding: None.

Conflict of Interest: The authors have o conflicts of interest to disclose.

References

1. Bader AG, Kang S, Zhao L, Vogt PK. Oncogenic PI3K deregulates transcription and translation. Nat Rev Cancer 2005; 5:921-929.
2. Hafner C, Landthaler M, Vogt T. Activation of the PI3K/AKT signalling pathway in non-melanoma skin cancer is not mediated by oncogenic PIK3CA and AKT1 hotspot mutations. Exp Dermatol 2010; 19:e222-7.
3. Kim YS, Shin S, Jung SH, Park YM, Park GS, Lee SH, Chung YJ. Genomic Progression of Precancerous Actinic Keratosis to Squamous Cell Carcinoma. J Invest Dermatol. 2022; 142(3 Pt A):528-538.e8.
4. Samuels Y, Velculescu VE. Oncogenic mutations of PIK3CA in human cancers. Cell Cycle. 2004; 3:1221-1224.
5. Janku F, Yap TA, Meric-Bernstam F. Targeting the PI3K pathway in cancer: Are we making headway? Nat Rev Clin Oncol. 2018; 15:273-291.

Received May 17, 2023; Revised August 21, 2023; Accepted August 24, 2023.

*Address correspondence to:
Ikko Kajihara, Department of Dermatology and Plastic Surgery, Faculty of Life Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto, Japan.
E-mail: kajiderma@gmail.com

Released online in J-STAGE as advance publication August 26, 2023.

IRDR
Intractable & Rare Diseases Research

Guide for Authors

1. Scope of Articles

Intractable & Rare Diseases Research (Print ISSN 2186-3644, Online ISSN 2186-361X) is an international peer-reviewed journal. *Intractable & Rare Diseases Research* devotes to publishing the latest and most significant research in intractable and rare diseases. Articles cover all aspects of intractable and rare diseases research such as molecular biology, genetics, clinical diagnosis, prevention and treatment, epidemiology, health economics, health management, medical care system, and social science in order to encourage cooperation and exchange among scientists and clinical researchers.

2. Submission Types

Original Articles should be well-documented, novel, and significant to the field as a whole. An Original Article should be arranged into the following sections: Title page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgments, and References. Original articles should not exceed 5,000 words in length (excluding references) and should be limited to a maximum of 50 references. Articles may contain a maximum of 10 figures and/or tables. Supplementary Data are permitted but should be limited to information that is not essential to the general understanding of the research presented in the main text, such as unaltered blots and source data as well as other file types.

Brief Reports definitively documenting either experimental results or informative clinical observations will be considered for publication in this category. Brief Reports are not intended for publication of incomplete or preliminary findings. Brief Reports should not exceed 3,000 words in length (excluding references) and should be limited to a maximum of 4 figures and/or tables and 30 references. A Brief Report contains the same sections as an Original Article, but the Results and Discussion sections should be combined.

Reviews should present a full and up-to-date account of recent developments within an area of research. Normally, reviews should not exceed 8,000 words in length (excluding references) and should be limited to a maximum of a maximum of 10 figures and/or tables and 100 references. Mini reviews are also accepted, which should not exceed 4,000 words in length (excluding references) and should be limited to a maximum of 5 figures and/or tables and 50 references.

Policy Forum articles discuss research and policy issues in areas related to life science such as public health, the medical care system, and social science and may address governmental issues at district, national, and international levels of discourse. Policy Forum articles should not exceed 3,000 words in length (excluding references) and should be limited to a maximum of 5 figures and/or tables and 30 references.

Communications are short, timely pieces that spotlight new research findings or policy issues of interest to the field of global health and medical practice that are of immediate importance. Depending on their content, Communications will be published as "Comments" or "Correspondence". Communications should not exceed 1,500 words in length (excluding references) and should be limited to a maximum of 2 figures and/or tables and 20 references.

Editorials are short, invited opinion pieces that discuss an issue of immediate importance to the fields of global health, medical practice, and basic science oriented for clinical application. Editorials should not exceed 1,000 words in length (excluding references) and should be limited to a maximum of 10 references. Editorials may contain one figure or table.

News articles should report the latest events in health sciences and medical research from around the world. News should not exceed 500 words in length.

Letters should present considered opinions in response to articles published in *Intractable & Rare Diseases Research* in the last 6 months or issues of general interest. Summaries of research results and sharing of experiences in clinical practice and basic research (findings based on case reports, clinical pictures, *etc*.) can also be published as Letters. Letters should not exceed 800 words in length and may contain a maximum of 10 references. Letters may contain one figure or table.

3. Editorial Policies

For publishing and ethical standards, *Intractable & Rare Diseases Research* follows the Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals issued by the International Committee of Medical Journal Editors (ICMJE, *https://icmje.org/recommendations*), and the Principles of Transparency and Best Practice in Scholarly Publishing jointly issued by the Committee on Publication Ethics (COPE, *https://publicationethics.org/resources/guidelines-new/principles-transparency-and-best-practice-scholarly-publishing*), the Directory of Open Access Journals (DOAJ, *https://doaj.org/apply/transparency*), the Open Access Scholarly Publishers Association (OASPA, *https://oaspa.org/principles-of-transparency-and-best-practice-in-scholarly-publishing-4*), and the World Association of Medical Editors (WAME, *https://wame.org/principles-of-transparency-and-best-practice-in-scholarly-publishing*).

Intractable & Rare Diseases Research will perform an especially prompt review to encourage innovative work. All original research will be subjected to a rigorous standard of peer review and will be edited by experienced copy editors to the highest standards.

Ethical Approval of Studies and Informed Consent: *F*or all manuscripts reporting data from studies involving human participants or animals, formal review and approval, or formal review and waiver, by an appropriate institutional review board or ethics committee is required and should be described in the Methods section. When your manuscript contains any case details, personal information and/or images of patients or other individuals, authors must obtain appropriate written consent, permission and release in order to comply with all applicable laws and regulations concerning privacy and/or security of personal information. The consent form needs to comply with the relevant legal requirements of your particular jurisdiction, and please do not send signed consent form to *Intractable & Rare Diseases Research* to respect your patient's and any other individual's privacy. Please instead describe the information clearly in the Methods (patient consent) section of your manuscript while retaining copies of the signed forms in the event they should be needed. Authors should also state that the study conformed to the provisions of the Declaration of Helsinki (as revised in 2013, *https://wma.net/what-we-do/medical-ethics/declaration-of-helsinki*). When reporting experiments on animals, authors should indicate whether the institutional and national guide for the care and use of laboratory animals was followed.

Reporting Clinical Trials: The ICMJE (*https:// icmje.org/recommendations/browse/publishing-and-editorial-issues/clinical-trial-registration.html*) defines a clinical trial as any research project that prospectively assigns people or a group of people to an intervention, with or without concurrent comparison or control groups, to study the relationship between a health-related intervention and a health outcome. Registration of clinical trials in a public trial registry at or before the time of first patient enrollment is a condition of consideration for publication in *Intractable & Rare Diseases Research*, and the trial registration number will be published at the end of the Abstract. The registry must be independent of for-profit interest and publicly accessible. Reports of trials must conform to CONSORT 2010 guidelines (*https://consort-statement.org/consort-2010*). Articles reporting the results of randomized trials must include the CONSORT flow diagram showing the progress of patients throughout the trial.

Conflict of Interest: All authors are required to disclose any actual or potential conflict of interest including financial interests or relationships with other people or organizations that might raise questions of bias in the work reported. If no conflict of interest exists for each author, please state "There is no conflict of interest to disclose".

Submission Declaration: When a manuscript is considered for submission to *Intractable & Rare Diseases Research*, the authors should confirm that 1) no part of this manuscript is currently under consideration for publication elsewhere; 2) this manuscript does not contain the same information in whole or in part as manuscripts that have been published, accepted, or are under review elsewhere, except in the form of an abstract, a letter to the editor, or part of a published lecture or academic thesis; 3) authorization for publication has been obtained from the authors' employer or institution; and 4) all contributing authors have agreed to submit this manuscript.

Initial Editorial Check: Immediately after submission, the journal's managing editor will perform an initial check of the manuscript. A suitable academic editor will be notified of the submission and invited to check the manuscript and recommend reviewers. Academic editors will check for plagiarism and duplicate publication at this stage. The journal has a formal recusal process in place to help manage potential conflicts of interest of editors. In the event that an editor has a conflict of interest with a submitted manuscript or with the authors, the manuscript, review, and editorial decisions are managed by another designated editor without a conflict of interest related to the manuscript.

Peer Review: *Intractable & Rare Diseases Research* operates a single-anonymized review process, which means that reviewers know the names of the authors, but the authors do not know who reviewed their manuscript. All articles are evaluated objectively based on academic content. External peer review of research articles is performed by at least two reviewers, and sometimes the opinions of more reviewers are sought. Peer reviewers are selected based on their expertise and ability to provide quality, constructive, and fair reviews. For research manuscripts, the editors may, in addition, seek the opinion of a statistical reviewer. Every reviewer is expected to evaluate the manuscript in a timely, transparent, and ethical manner, following the COPE guidelines (*https://publicationethics.org/files/cope-ethical-guidelines-peer-reviewers-v2_0.pdf*). We ask authors for sufficient revisions (with a second round of peer review, when necessary) before a final decision is made. Consideration for publication is based on the article's originality, novelty, and scientific soundness, and the appropriateness of its analysis.

Suggested Reviewers: A list of up to 3 reviewers who are qualified to assess the scientific merit of the study is welcomed. Reviewer information including names, affiliations, addresses, and e-mail should be provided at the same time the manuscript is submitted online. Please do not suggest reviewers with known conflicts of interest, including participants or anyone with a stake in the proposed research; anyone from the same institution; former students, advisors, or research collaborators (within the last three years); or close personal contacts. Please note that the Editor-in-Chief may accept one or more of the proposed reviewers or may request a review by other qualified persons.

Language Editing: Manuscripts prepared by authors whose native language is not English should have their work proofread by a native English speaker before submission. If not, this might delay the publication of your manuscript in *Intractable & Rare Diseases Research*.

The Editing Support Organization can provide English proofreading, Japanese-English translation, and Chinese-English translation services to authors who want to publish in *Intractable & Rare Diseases Research* and need assistance before submitting a manuscript. Authors can visit this organization directly at *https://www.iacmhr.com/iac-eso/support.php?lang=en*. IAC-ESO was established to facilitate manuscript preparation by researchers whose native language is not English and to help edit works intended for international academic journals.

Copyright and Reuse: Before a manuscript is accepted for publication in *Intractable & Rare Diseases Research*, authors will be asked to sign a transfer of copyright agreement, which recognizes the common interest that both the journal and author(s) have in the protection of copyright. We accept that some authors (e.g., government employees in some countries) are unable to transfer copyright. A JOURNAL PUBLISHING AGREEMENT (JPA) form will be e-mailed to the authors by the Editorial Office and must be returned by the authors by mail, fax, or as a scan. Only forms with a hand-written signature from the corresponding author are accepted. This copyright will ensure the widest possible dissemination of information. Please note that the manuscript will not proceed to the next step in publication until the JPA Form is received. In addition, if excerpts from other copyrighted works are included, the author(s) must obtain written permission from the copyright owners and credit the source(s) in the article.

4. Cover Letter

The manuscript must be accompanied by a cover letter prepared by the corresponding author on behalf of all authors. The letter should indicate the basic findings of the work and their significance. The letter should also include a statement affirming that all authors concur with the submission and that the material submitted for publication has not been published previously or is not under consideration for publication elsewhere. The cover letter should be submitted in PDF format. For an example of Cover Letter, please visit: (Download Centre: *https://www.irdrjournal.com/downcentre*)

5. Submission Checklist

The Submission Checklist should be submitted when submitting a manuscript through the Online Submission System. Please visit Download Centre (*https://www.irdrjournal.com/downcentre*) and download the Submission Checklist file. We recommend that authors use this checklist when preparing your manuscript to check that all the necessary information is included in your article (if applicable), especially with regard to Ethics Statements.

6. Manuscript Preparation

Manuscripts are suggested to be prepared in accordance with

the "Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals", as presented at *https://www.ICMJE.org*.

Manuscripts should be written in clear, grammatically correct English and submitted as a Microsoft Word file in a single-column format. Manuscripts must be paginated and typed in 12-point Times New Roman font with 24-point line spacing. Please do not embed figures in the text. Abbreviations should be used as little as possible and should be explained at first mention unless the term is a well-known abbreviation (*e.g*. DNA). Single words should not be abbreviated.

Title page: The title page must include 1) the title of the paper (Please note the title should be short, informative, and contain the major key words); 2) full name(s) and affiliation(s) of the author(s), 3) abbreviated names of the author(s), 4) full name, mailing address, telephone/fax numbers, and e-mail address of the corresponding author; 5) author contribution statements to specify the individual contributions of all authors to this manuscript, and 6) conflicts of interest (if you have an actual or potential conflict of interest to disclose, it must be included as a footnote on the title page of the manuscript; if no conflict of interest exists for each author, please state "There is no conflict of interest to disclose").

Abstract: The abstract should briefly state the purpose of the study, methods, main findings, and conclusions. For articles that are Original Articles, Brief Reports, Reviews, or Policy Forum articles, a one-paragraph abstract consisting of no more than 250 words must be included in the manuscript. For Communications, Editorials, News, or Letters, a brief summary of main content in 150 words or fewer should be included in the manuscript. For articles reporting clinical trials, the trial registration number should be stated at the end of the Abstract. Abbreviations must be kept to a minimum and non-standard abbreviations explained in brackets at first mention. References should be avoided in the abstract. Three to six key words or phrases that do not occur in the title should be included in the Abstract page.

Introduction: The introduction should provide sufficient background information to make the article intelligible to readers in other disciplines and sufficient context clarifying the significance of the experimental findings.

Materials/Patients and Methods: The description should be brief but with sufficient detail to enable others to reproduce the experiments. Procedures that have been published previously should not be described in detail but appropriate references should simply be cited. Only new and significant modifications of previously published procedures require complete description. Names of products and manufacturers with their locations (city and state/country) should be given and sources of animals and cell lines should always be indicated. All clinical investigations must have been conducted in accordance with Declaration of Helsinki principles. All clinical investigations must have been conducted in accordance with the Declaration of Helsinki (as revised in 2013, *https://wma.net/what-we-do/medical-ethics/declaration-of-helsinki*). All human and animal studies must have been approved by the appropriate institutional review board(s) and a specific declaration of approval must be made within this section.

Results: The description of the experimental results should be succinct but in sufficient detail to allow the experiments to be analyzed and interpreted by an independent reader. If necessary, subheadings may be used for an orderly presentation. All Figures and Tables should be referred to in the text in order, including those in the Supplementary Data.

Discussion: The data should be interpreted concisely without repeating material already presented in the Results section. Speculation is permissible, but it must be well-founded, and discussion of the wider implications of the findings is encouraged. Conclusions derived from the study should be included in this section.

Acknowledgments: All funding sources (including grant identification) should be credited in the Acknowledgments section. Authors should also describe the role of the study sponsor(s), if any, in study design; in the collection, analysis, and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication. If the funding source had no such involvement, the authors should so state.

In addition, people who contributed to the work but who do not meet the criteria for authors should be listed along with their contributions.

References: References should be numbered in the order in which they appear in the text. Citing of unpublished results, personal communications, conference abstracts, and theses in the reference list is not recommended but these sources may be mentioned in the text. In the reference list, cite the names of all authors when there are fifteen or fewer authors; if there are sixteen or more authors, list the first three followed by *et al*. Names of journals should be abbreviated in the style used in PubMed. Authors are responsible for the accuracy of the references. The EndNote Style of *Intractable & Rare Diseases Research* could be downloaded at **EndNote** (*https://www.irdrjournal.com/examples/Intractable_Rare_Diseases_Research.ens*).

Examples are given below:

Example 1 (Sample journal reference):

Inagaki Y, Tang W, Zhang L, Du GH, Xu WF, Kokudo N. Novel aminopeptidase N (APN/CD13) inhibitor 24F can suppress invasion of hepatocellular carcinoma cells as well as angiogenesis. Biosci Trends. 2010; 4:56-60.

Example 2 (Sample journal reference with more than 15 authors):

Darby S, Hill D, Auvinen A, *et al*. Radon in homes and risk of lung cancer: Collaborative analysis of individual data from 13 European case-control studies. BMJ. 2005; 330:223.

Example 3 (Sample book reference):
Shalev AY. Post-traumatic stress disorder: Diagnosis, history and life course. In: Post-traumatic Stress Disorder, Diagnosis, Management and Treatment (Nutt DJ, Davidson JR, Zohar J, eds.). Martin Dunitz, London, UK, 2000; pp. 1-15.

Example 4 (Sample web page reference):

World Health Organization. The World Health Report 2008 – primary health care: Now more than ever. *http://www.who.int/whr/2008/whr08_en.pdf* (accessed September 23, 2022).

Tables: All tables should be prepared in Microsoft Word or Excel and should be arranged at the end of the manuscript after the References section. Please note that tables should not in image format. All tables should have a concise title and should be numbered consecutively with Arabic numerals. If necessary, additional information should be given below the table.

Figure Legend: The figure legend should be typed on a separate page of the main manuscript and should include a short title and explanation. The legend should be concise but comprehensive and should be

understood without referring to the text. Symbols used in figures must be explained. Any individually labeled figure parts or panels (A, B, *etc*.) should be specifically described by part name within the legend.

Figure Preparation: All figures should be clear and cited in numerical order in the text. Figures must fit a one- or two-column format on the journal page: 8.3 cm (3.3 in.) wide for a single column, 17.3 cm (6.8 in.) wide for a double column; maximum height: 24.0 cm (9.5 in.). Please make sure that the symbols and numbers appeared in the figures should be clear. Please make sure that artwork files are in an acceptable format (TIFF or JPEG) at minimum resolution (600 dpi for illustrations, graphs, and annotated artwork, and 300 dpi for micrographs and photographs). Please provide all figures as separate files. Please note that low-resolution images are one of the leading causes of article resubmission and schedule delays.

Units and Symbols: Units and symbols conforming to the International System of Units (SI) should be used for physicochemical quantities. Solidus notation (*e.g*. mg/kg, mg/mL, mol/mm^2/min) should be used. Please refer to the SI Guide *www.bipm.org/en/si/* for standard units.

Supplemental data: Supplemental data might be useful for supporting and enhancing your scientific research and *Intractable & Rare Diseases Research* accepts the submission of these materials which will be only published online alongside the electronic version of your article. Supplemental files (figures, tables, and other text materials) should be prepared according to the above guidelines, numbered in Arabic numerals (*e.g*., Figure S1, Figure S2, and Table S1, Table S2) and referred to in the text. All figures and tables should have titles and legends. All figure legends, tables and supplemental text materials should be placed at the end of the paper. Please note all of these supplemental data should be provided at the time of initial submission and note that the editors reserve the right to limit the size and length of Supplemental Data.

7. Online Submission

Manuscripts should be submitted to *Intractable & Rare Diseases Research* online at *https://www.irdrjournal.com*. Receipt of your manuscripts submitted online will be acknowledged by an e-mail from Editorial Office containing a reference number, which should be used in all future communications. If for any reason you are unable to submit a file online, please contact the Editorial Office by e-mail at *office@irdrjournal.com*

8. Accepted Manuscripts

Proofs: Galley proofs in PDF format will be sent to the corresponding author *via* e-mail. Corrections must be returned to the editor (*office@irdrjournal.com*) within 3 working days.

Offprints: Authors will be provided with electronic offprints of their article. Paper offprints can be ordered at prices quoted on the order form that accompanies the proofs.

Page Charge: No page charges will be levied to authors for the publication of their article except for reprints.

Misconduct: *Intractable & Rare Diseases Research* takes seriously all allegations of potential misconduct and adhere to the ICMJE Guideline (*https://icmje.org/recommendations*) and COPE Guideline (*http://publicationethics.org/files/Code_of_conduct_for_journal_editors.pdf*). In cases of suspected research or publication misconduct, it may be necessary for the Editor or Publisher to contact and share submission details with third parties including authors' institutions and ethics committees. The corrections, retractions, or editorial expressions of concern will be performed in line with above guidelines.

(As of December 2022)

Intractable & Rare Diseases Research
Editorial and Head Office
Pearl City Koishikawa 603,
2-4-5 Kasuga, Bunkyo-ku,
Tokyo 112-0003, Japan.
E-mail: office@irdrjournal.com