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Editorial Board

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

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Review

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Global frailty screening tools: Review and application of frailty screening tools from 2001 to 2023

Yi Deng[1,*], Naomi Sato[2]

[1] Graduate School of Nursing, Hamamatsu University School of Medicine, Hamamatsu, Shizuoka, Japan;
[2] Department of Clinical Nursing, Hamamatsu University School of Medicine, Hamamatsu, Shizuoka, Japan.

SUMMARY As the aging population increases globally, health-related issues caused by frailty are gradually coming to light and have become a global health priority. Frailty leads to a significantly increased risk of falls, incapacitation, and death. Early screening leads to better prevention and management of frailty, increasing the possibility of reversing it. Developing assessment tools by incorporating disease states of older adults using effective interventions has become the most effective approach for preventing and controlling frailty. The most direct and effective tool for evaluating debilitating conditions is a frailty screening tool, but because there is no globally recognized gold standard, every country has its own scale for national use. The diversity and usefulness of the frailty screening tool has become a hot topic worldwide. In this article, we reviewed the frailty screening tool published worldwide from January 2001 to June 2023. We focused on several commonly used frailty screening tools. A systematic search was conducted using PubMed database, and the commonly used frailty screening tools were found to be translated and validated in many countries. Disease-specific scales were also selected to fit the disease. Each of the current frailty screening tools are used in different clinical situations, and therefore, the clinical practice applications of these frailty screening tools are summarized graphically to provide the most intuitive screening and reference for clinical practitioners. The frailty screening tools were categorized as (*i*) Global Frailty Screening Tools in Common; (*ii*) Frailty Screening Tools in various countries; (*iii*) Frailty Screening Tools for various diseases. As science and technology continue to advance, electronic frailty assessment tools have been developed and utilized. In the context of Coronavirus disease 2019 (COVID-19), electronic frailty assessment tools played an important role. This review compares the currently used frailty screenings tools, with a view to enable quick selection of the appropriate scale. However, further improvement and justification of each tool is needed to guide clinical practitioners to make better decisions.

Keywords frailty, screening tools, early screening, aging, electronic frailty assessment tool

1. Introduction

Frailty is a complex clinical syndrome with multiple causes and contributing factors. Frailty leads to increased vulnerability to minor stress triggers and increased risk for adverse outcomes, such as disability, hospitalization and mortality (*1,2*). It is often manifested by a maladaptive response to stress triggers, leading to a vicious cycle toward functional decline and other serious adverse health outcomes. Frailty is characterized by diminished strength, endurance and physiological reserve across the neuromuscular, metabolic and immune systems (*3*), becomes more prevalent with age, imposing substantial burdens on patients and caregivers (*4*). Notably, old age itself does not define frailty because some patients are active despite advanced age, whereas others can have a functional decline in the absence of apparent stress factors or failure to rebound following hospitalization or illness (*5*). As a dynamic and reversible geriatric syndrome, it has become one of the important public health problems emerging around the world (*6*).

Frailty can affect anyone during all stages of life, and has a prevalence rate from 4 to 59.1% based on various demographic or socioeconomic conditions (*7*). A meta-analysis from China showed the prevalence of frailty in adults aged ≥ 5 0 years was 12-24% worldwide. Its prevalence ranged from 6-25% in adults aged 65-74 years, and an average of 10% in adults aged ≥ 85 years. Using a common frailty assessment instrument, an estimated 15% of non-institutionalized adults in the

United States are frail (*8*), and global estimates of frailty range from 3.5-27.3% (*9*). In Canada, about 25% of the population aged over 65 years are frail, and over 50% of the population aged ≥ 85 years are frail (*10*). Research has approximated that 10% of community dwelling older adults are considered frail and over 41% considered pre-frail in Canada (*11*). In Australia, frailty is estimated to range between 4.9 to 27.3%, depending on the region and the measurement instrument used, and the prevalence of pre-frailty– a "clinically silent" intermediate stage between non-frail/robust and frail ranged from 34.6 and 50.9% (*12*). Like many countries, Australia's population of older adults is rapidly increasing. It has been estimated that by 2031, 3.9 million Australians aged ≥ 65 years will be either be frail or at-risk of becoming frail (*13*). In Spain, for the community-dwelling population aged ≥ 65 years, frailty prevalence has been estimated to be 11%, and that of pre-frailty is estimated to be 35-40%. Prevalence increases with age and can reach as high as 50% in the population aged > 80 years, and is slightly higher in women than in men (*14*). Another study using physical frailty models in adults aged ≥ 50 years old from 62 countries showed that the highest prevalence of physical frailty was found in Africa (22%) and the lowest prevalence was in Europe (8%), while the pre-frailty prevalence was highest in the Americas (50%) and lowest in Europe (42%). However, using deficit accumulation models, the prevalence of frailty was found to be highest in Oceania (31%) and lowest in Europe (22%), while pre-frailty prevalence was highest in Oceania (51%) and lowest in Europe and Asia (49%). The population-level frailty prevalence among community-dwelling adults varied by age, sex, and frailty classification (*15*). This shows that frailty-related debilitation is a global problem which cannot be ignored, and the prevalence of debility varies from one debilitating assessment tool to another. However, there is no international consensus for a common definition of frailty. For that reason, many tools have been developed over the years to identify, measure and assess frailty.

2. Common global frailty screening tools

In the past decade, numerous tools have been developed to screen or assess frailty. Since the release of the "Fried Frailty Phenotype" (FP) scale by Fried *et al*. (*16*) in 2001, nearly 70 frailty scales have been developed to identify frailty with various aspects of physical, psychological, or social components. Nonetheless, each screening scale has its advantages in the disease setting for which it was developed, and the differences in the selection and application of debilitating scales are apparent and cannot be ignored. However, there is no standard assessment instrument. The most widely used measures are the FP (*16*) and the frailty index (FI), but these cannot be easily implemented in large-scale population studies or busy clinics (*17*). To assess frailty in large populations, it is important to find short-term rapid instruments that give reliable results for the risk of a negative event and to stratify older adults according to their level of frailty. In this way the most appropriate strategies can be chosen and applied to delay the functional decline associated to frailty and its consequences, such as hospitalization, institutionalization, low quality of life, and death (*18*). The common debilitation assessment scales provided by national and international literature and consensus of relevant guidelines are presented in Table 1.

2.1. Fried's Frailty Phenotype

The Fried's Frailty Phenotype (FP) tool proposed by Fried *et al*. (*16*) includes five items: unintentional weight loss (4.5 kg or more over the past year), exhaustion (self-reported), low physical activity, weakness (low grip strength), and walking speed. Individuals with two deficits were considered pre-frail, and those with three or more deficits were classified as frail. This tool is based on the biological causative theory and it is predictive of adverse clinical outcomes. Although this tool should be able to identify frailty and to predict adverse outcomes and is widely used in clinical and research settings (*19*), it requires the measurement of grip strength, which is not usually done in medical activities.

2.2. Groningen Frailty Indicator

The Groningen Frailty Indicator (GFI) (*20*) is a 15-item screening instrument to determine the level of frailty, which is available as a specialized and self-reported version. It measures the loss of functions and resources in four domains: physical (mobility functions, multiple health problems, physical fatigue, vision, hearing), cognitive (cognitive dysfunction), social (emotional isolation), and psychological (depressed mood and feelings of anxiety). The range of the GFI score is 0 to 15. Geriatric experts agreed that a score of 4 or higher represented moderate to severe frailty. Major drawbacks of most of these instruments are that they (*i*) assess frailty in specific older adult populations only (*e.g*., home-dwelling), (*ii*) do not contain a specialized and a self-assessed version, (*iii*) do not comprise of items that assess disability that could predict poor outcome, and (*iv*) do not allow for grades of frailty to be identified (*21*). The GFI (*20*) is an instrument that includes all these domains and meets the drawbacks of other instruments. The GFI is widely used in clinical practice (*i.e.*, geriatric centers, nursing homes, emergency departments, traumatology, pulmonology, rheumatology, and surgical medicine), in outpatient settings, and in clinical studies (*21*).

2.3. Frailty Index

The Frailty Index (FI) by Mitnitski *et al*. (*22*) was

Table 1. Global frailty screening tools in common

Title/Year	Country	Publishers	Items	Components	Frailty classification
Frailty Phenotype (FP) / 2001	US	Fried *et al.* (*16*)	5	It assesses physical characteristics or phenotype, which include five domains: unintentional weight loss (4.5 kg or more in the last year), exhaustion (self-reported), low physical activity, weakness (low grip strength), and walking speed.	Frailty: ≥ 3 items; pre-frailty:1~2 items; robust:0 items
Groningen Frailty Indicator (GFI) / 2001	Netherlands	Steverink *et al.* (*20*)	15	Physical (9 items), Cognitive (1 item), Social (3 items), and Psychological (2 items), for a total of 4 dimensions.	Frailty: score ≥ 4
Frailty Index (FI) / 2001	Canadian	Mitnitski *et al.* (*22*)	30~70	all the 8 frailty items and all the 3 domains (physical, psychological and social) are assessed.	Frailty: score > 0.25; pre-frailty:0.12~0.25; robust: score < 0.12
Clinical Frailty Scale (CFS) / 2005	Canadian	Rockwood *et al.* (*28*)	9	Total 9 points: each point on its scale has a visual chart and a written description of frailty to assist the classification process.	Frailty: score ≥ 5
Edmonton Frail Scale (EFS) / 2006	Canadian	Rolfson *et al.* (*29*)	11	The EFS is an 11-item scale, of which 9 items are self-reported, It is assesses nine domains of frailty (cognition, general health status, functional independence, social support, medication usage, nutrition, mood, continence, functional performance).	The following cut-offs are used to classify frailty severity: not frailty (0-5), apparently vulnerable (6-7), mildly frailty (8-9), moderately frailty (10-11) and severely frailty
The Tilburg Frailty Indicator (TFI) / 2010	Netherlands	Gobbens *et al.* (*31*)	15	The TFI is composed of 2 parts: Part A about "determinants of frailty and diseases", and Part B about the "presence of frailty" that generates a final score. Part B includes three domains (physical, psychological, and social) and 15 items.	Frailty: score ≥ 5

developed using the Canadian Study of Health and Aging (CSHA) data. It is a continuous scoring system where eight frailty items and three domains (physical, psychological, and social) are assessed. This tool evaluates the presence of health deficits (*e.g.*, comorbidities, symptoms, disabilities, and diseases). Although the Frailty Index can be used by clinicians in hospitals and in community settings and by researchers, it is not easy to use because it involves mathematical calculations (*19*). Hence, Sternberg *et al*. (*23*) had proposed this tool to plan health services.

2.4. Clinical Frailty Scale

The Clinical Frailty Scale (CFS) is a clinical judgement-based frailty tool developed for the Canadian Study of Health and Aging (*24*). The CFS evaluates specific domains including comorbidity, function, and cognition to generate a frailty score ranging from 1 (very fit) to 9 (terminally ill). Various reviews have been published on frailty using the CFS tool indicating it to be a promising frailty screening tool (*25-27*). The CFS has been used in a variety of contexts around the world. Although most administered in Canada and the United Kingdom, this frailty tool is also used in Asia, South America, and other parts of Europe. The CFS is most often used in hospital settings in several inpatient and outpatient populations, particularly in geriatric e and cardiology units. The increase of its use in a variety of settings shows that researchers and clinicians value the ease and efficiency this judgement-based tool. In research, the CFS is commonly used to predict health outcomes, mostly mortality, comorbidity, functional decline, mobility, and cognitive decline (*28*).

2.5. Edmonton Frail Scale

The Edmonton Frail Scale (EFS) (*29*) is an 11-item scale, of which nine items are self-reported. It assesses nine domains of frailty (cognition, general health status, functional independence, social support, medication usage, nutrition, mood, continence, functional performance). Test results can be from 0 to 17. The participants are classified conventionally into three categories, and a higher score represents a higher degree of fragility. Severe Frail and non-frail participants were defined according to the EFS score as "no frailty" (≤ 5 points), "apparently vulnerable" ($6 \leq n \geq 11$ points), and "severe frailty" ($12 \leq n \geq 17$ points). Of note, the EFS was validated by non-specialists who had no formal training in geriatric care. The administration of the EFS questionnaire requires 3-5 min. Thus, the EFS can be a practical and clinically meaningful measure of frailty in a variety of settings. Perna *et al.* (*30*) suggested that EFS is a helpful tool to stratify the state of frailty in a group of institutionalized older individuals. As matter of fact, the EFS has been shown to be associated with several

geriatric conditions such independence, drugs assumption, mood, mental, functional and nutritional status.

2.6. Tilburg Frailty Indicator

The Tilburg Frailty Indicator (TFI) (*31*) is a self-reported scale. It was proposed in 2010, and it is regularly used in the context of community-dwelling older people. Besides, over the past years since its introduction, the TFI scale has been widely used in research and has been translated into many languages (including Italian). This tool is a self-administered questionnaire and evaluates all physical, social, and psychological domains. The Tilburg Frailty Indicator questionnaire administration requires 14 min and measures six criteria for quality on a scale of 1–10 (*32,33*). The TFI is composed of two parts, part A evaluates "determinants of frailty and diseases", and part B about the "presence of frailty" that generates a final score. Part B includes three domains (physical, psychological, and social) and a total of 15 items. A total score ≥ 5 is set as the threshold for frailty. The TFI Part B is a self-reported scale used in several countries, and it is associated with short-term disability, lower quality of life, hospitalization, and falls (*32*). The TFI needs further evaluation in larger studies (*33,34*), even though the tool has been evaluated for almost all psychometric domains and shows good validity and reliability for the PHC setting (*33,34*) and the physical items present a good predictive ability of adverse outcomes (*32*).

3. Frailty screening tools in various countries

Every country chooses a debilitation scale that is appropriate for use in its own country. Debilitation scales used in various countries are shown in Table 2. Each country also validates commonly used debilitation scales and develops a debilitation scale as required. In recent years, a Chinese self-reported frailty screening questionnaire (FSQ) based on modified Fried FP criteria was developed and validated in different settings (*35*). A Chinese version of the TFI was also developed to measure frailty among community-dwelling older adults (*36*). A 10-item Chinese frailty screening scale (CFSS-10) (*17*) was successfully developed and validated. The CFSS-10 has good validity and reliability as a quick and acculturative frailty screening scale for community-dwelling older adults in Shanghai. It might also supplement the existing frailty screening tools. In 2006, the Japanese government implemented the Long-Term Care Insurance (LTCI) system with the introduction of preventive care and the improvement of quality of care. The LTCI system uses the Kihon Checklist (KCL), a self-reported comprehensive health checklist designed by a study group from the MHLW, as a screening tool to identify community-dwelling older adults who were vulnerable to frailty (*37*). The CFS is a valid, reliable and easy-to-use tool that has been translated in several languages. A Greek version of the revised nine-scale CFS is a valid and reliable instrument for the identification of frailty in Greek population (*38*). Our academic hospital-based study used the Thai-language version of the Frailty Assessment Tool (Thai Ministry of Public Health) and the Frail Non-Disabled (FiND) questionnaire and showed that the two scales had slight to moderate agreement with Fried's Frailty Phenotype (FFP). Additionally, their predictive power was low and, thus, insufficient for frailty detection in a clinical setting. Further research in a multicenter setting of these and other assessment tools is needed to improve frailty screening in older Thai populations (*15*). In Korea, the Korean Frailty Index (KFI) and the modified KFI (mKFI) are valid instruments for frailty screening and might be useful as simple frailty screening tools to identify older adults who might benefit from comprehensive geriatric assessment and integrated, multidisciplinary geriatric care services (*39*).

4. Frailty screening tools for various diseases

Due to the complexity and specificity of the tools, a wide variety of tools are available for assessment and screening for different diseases (Table 3).

4.1. Cardiovascular diseases (CVD)

Frailty is an important prognostic factor in patients with cardiovascular diseases (CVD), and so identifying frailty in these patients might help to tailor the cardiovascular treatment to these individuals. The first step is to identify frailty. Several tools have been validated as screening methods for frailty. However, to the tools vary in complexity, nature, feasibility and the outcomes that can be predicted (*40*). An ideal frailty screening tool should (*i*) be able to accurately identify frailty, (*ii*) predict the response of frail patients to potential therapies, and (*iii*) be simple and easy to apply and have low cost (*41*). For CVD, the two most used and robust frailty assessment tools for clinicians and researchers are the Fried criteria and frailty indices (*40*). In addition to these two screening tools, other screening tools can also be used to evaluate the cardiovascular system. Kang *et al*. used the CFS for assessment of acute coronary syndrome (ACS) in older patients to predict all-cause mortality, unscheduled return visit, and in-hospital and recurrent major adverse cardiovascular events (*42*). Boxer *et al*. also found that the six-minute walk and the five-item Cardiovascular Health Study were independently predictive of mortality in older adults with heart failure, with hazard ratio (HR) 0.82 and 1.64, respectively, and these were a useful measure of frailty (*43*). However, there is no optimal assessment method for debilitating cardiovascular diseases.

4.2. Cancer

The incidence of frailty in older cancer patients is

Table 2. Frailty screening tools in various countries

US (*71*)	1) The Brief Risk Identification of Geriatric Health Tool (BRIGHT Tool); 2) Frailty Index; 3) Fried Phenotype; 4) The Gerontopole Frailty Screening Tool; 5) Groningen Frailty Index; 6) The PRISMA-7; 7) Simple Fatigue, Resistance, Ambulation, Illnesses, & Loss of Weight (FRAIL) Scale; 8) Strawbridge Questionnaire; 9) Tilburg Frailty Indicator.
UK (*72-74*)	1) The Fried Frailty Phenotype; 2) The FRAIL scale; 3) General Medical Services (GMS); 4) Short physical performance battery (SPPB); 5) The Clinical Frailty Scale (CFS); 6) The PRISMA-7; 7) The electronic Frailty Index (eFI); 8) The Frailty Index (FI).
Canada (*10,11,75*)	1) The frailty phenotype model; 2) The Clinical Frailty Scale (CFS); 3) The Edmonton Frail Scale; 4) Groningen Frailty Indicator (GFI); 5) Tilburg Frailty Indicator; 6) PRISMA 7, gait speed, hand grip strength, balance testing and Timed Up and Go; 7) A comprehensive geriatric assessment (CGA); 8) The Assessment Urgency Algorithm (AUA); 9) Seniors Fitness Test (SFT); 10) Short physical performance battery (SPPB); 11) The Tilburg Frailty Indicator (TFI); 2) the Sherbrooke Postal Questionnaire (SPQ).
French (*76*)	1) The Study of Osteoporotic Fractures (SOF) index; 2) The Fried frailty phenotype.
Italian (*77*)	1) The Italian Frailty index (IFi); 2) The Comprehensive Geriatric Assessment (CGA); 3) The AGILE; 4) The Clinical Frailty Scale (CFS); 5) The Study of Osteoporotic Fractures (SOF) index; 6) The FRAIL index; 7) The Tilburg Frailty Indicator (TFI); 8) The PRISMA-7.
Netherlands (*20,78*)	1) The 13-item RISK scale; 2) The validated 15-item Groningen Frailty Indicator (GFI); 3) The validated Maastricht Frailty Screening Tool for Hospitalized Patients (MFST-HP); 4) The PERSSILAA.
Spain (*79*)	1) Timed up and go test (TUG); 2) Short Physical Performance battery (SPPB); 3) Tilburg Frailty Indicator (TFI); 4) The Gerontopole Frailty Scale (GFS).
Japan (*80,81*)	1) The phenotype model; 2) The defcit accumulation model; 3) The Kihon Checklist (KCL); 4) Frailty screening index (FSI); 5) A Japan frailty scale (JFS).
China (*17,20,82,83*)	1) A 10-item Chinese frailty screening scale (CFSS-10); 2) The Tilburg frailty indictor (TFI); 3) A 49-item Frailty Index (FI); 4) The Fried frailty phenotype (FP); 5) A Chinese self-reported frailty screening questionnaire (FSQ); 6) The FI-35; 7) A Chinese version of the Tilburg frailty indictor (TFI); 8) A Japan frailty scale (JFS); 9) Multidisciplinary teams (MDT); 10) Clinical Frailty Scale (CFS); 11) FRAIL scale; 12) The Edmonton frail scale; 13) The comprehensive geriatric assessment – frailty index (CGA-FI); 14) The combined index; 15) The Chinese version of Trauma-Specific Frailty Index(C-TSFI).
Singapore (*84,85*)	1) Frail-PPS (Frail-Physical, Psychological and Social); 2) Frailty Assessment Measure (FAM); 3) Identification of seniors at-risk hospitalized patients (ISAR-HP); 4) The frailty phenotype; 5) The deficit accumulation models; 6) Asia-Pacific Clinical Practice Guidelines for the Management of Frailty (AP-CPGMF); 7) The Comprehensive Geriatric Assessment (CGA); 8) The Edmonton frail scale; 9) The Frailty Index (FI).
Denmark (*86*)	1) Fried's Phenotype (FP); 2) The Clinical Frailty Scale (CFS).
Greek (*38*)	1) The Clinical Frailty Scale (CFS).
African (*87*)	1) The Fried frailty phenotype (FFP); 2) The Clinical Frailty Scale (CFS); 3) Brief Frailty Instrument for Tanzania (B-FIT 2).
Thai (*15,88*)	1) The Thai version of the Simple Frailty Questionnaire (T- FRAIL); 2) The Thai Frailty Index (TFI); 3) The Frailty Assessment Tool of the Thai Ministry of Public Health (FATMPH); 4) The Frail Non-Disabled (FiND) questionnaire; 5) Fried's Frailty Phenotype (FFP); 6) The Clinical Frailty Scale (CFS); 7) The PRISMA-7 questionnaire; 8) The Timed Up and Go (TUG) test; 9) The Gerontopole frailty screening tool (GFST).
Korean (*39,89*)	1) The Korean Frailty Index (KFI); 2) The modified KFI (mKFI); 3) Cardiovascular Health Study (CHS); 4) Comprehensive geriatric assessment (CGA); 5) The timed up and go (TUGT) test; 6) The short physical performance battery (SPPB); 7) The Clinical Frailty Scale (CFS); 8) The Korean version of FRAIL (K-FRAIL); 9) Korean Cancer Study Group Geriatric Score (KG-7); 10) Korean Frailty Index; 11) The Korean version of the CSF (CSF-K); 12) The Eastern Cooperative Oncology Group Performance Status (ECOG PS) scale.

significantly higher than that in people of the same age (*44*). Frailty assessments can detect more health problems, prevent function deterioration, and determine the most feasible cancer. Frailty assessment is also vital in deciding if a patient would benefit from the proposed treatment. Therefore, it is necessary to assess the degree of frailty in older patients with cancer to optimize personalized care strategies (*45*). Comprehensive Geriatric Assessment (CGA) is the gold standard for detecting frailty in older patients with cancer. Since CGA is time- and resource-consuming, many alternative frailty screening tools have been developed; however, it remains unknown whether these tools are suitable for older and adult patients with cancer (*46*). Both the Geriatric 8 questionnaire (G8) and the Korean Cancer Study Group Geriatric Score (KG-7) were designed to screen for frailty in older patients with cancer. The KG-7 is a novel geriatric screening tool. Shorter screening tools can identify patients who might benefit from a full Geriatric assessment (GA) (*45*).

4.3. Nephrology

There is a correlation between kidney-like diseases and

Table 3. Frailty screening tools for various diseases

Cardiovascular disease (CVD) (*40*)	1) The Clinical Frailty Scale (CFS); 2) The Tilburg Frailty Indicator (TFI) and its mental and physical domains; 3) The six-minute walk and the five-item Cardiovascular Health Study; 4) The seven-item Cardiovascular Health Study score, the Short Physical Performance Battery (SPPB) and a 35-item frailty index; 5) CAF; 6) Modified Fried frailty criteria; 7) 4 scales used:5-item Modified Fried Criteria; 7-item expanded Modified Fried Criteria; 4-item MSSA;Five-Meter Gait Speed Test; 8) Multidimensional Geriatric Assessment; 9) Geriatric baseline examination; 10) 31-item deficit index; 11) Essential Frailty Toolset.
Cancer (*45,47,90,91*)	1) Geriatric assessment (GA); 2) The Groningen Frailty Indicator (GFI); 3) The Vulnerable Elders Survey-13 (VES-13); 4) The Geriatric 8 (G8) questionnaire; 5) The Korean Cancer Study Group Geriatric Score (KG-7); 6) Flemish version of the Triage Risk Screening Tool (fTRST); 7) The modifed frailty index score (mFI-5); 8) The frailty phenotype; 9) The accumulated deficits theories; 10) The comprehensive geriatric assessment (CGA); 11) The Eastern Cooperative Oncology Group Performance Status (ECOG PS).
Nephrology (*47,49,50,92*)	1) The Fried Phenotype; 2) The Clinical Frailty Scale (CFS); 3) The Frailty Index (FI); 4) Self-rated health (SRH); 5) The surprise question (SQ); 6) The Clinical Frailty Scale (CFS); 7) A Comprehensive Geriatric Assessment (CGA); 8) The Vulnerable Elders Survey-13 (VES-13); 9) The Geriatric 8 (G8) questionnaire.
Cirrhosis (*51,93*)	1) The Fried Frailty Index (FFI); 2) The Clinical Frailty Scale (CFS) and the Montreal Cognitive Assessment (MoCA); 3) The Short Physical Performance Battery (SPPB); 4) The Liver Frailty Index (LFI); 5) Timed-up-and-go test (TUG); 6) The Liver Frailty Index; 7) Combining grip strength, chair stands (CST) and balance tests; 8) The frailty phenotype; 9) Fried Frailty Criteria (FFC); 10) 6-minute walk test (6MWT); 11) Activities of Daily Living (ADL); 12) Cardiopulmonary exercise testing (CPET); 13) Gait speed; 14) Grip strength; 15) Instrumental Activities of Daily Living (IADL); 16) Karnofsky Performance Status (KPS).
Chronic obstructive pulmonary disease (COPD) (*56*)	1) The Fried frailty phenotype (FFP); 2) The Clinical Frailty Scale (CFS); 3) Frailty Index of Accumulative Defcits (FI-CD); 4) The Short Physical Performance Battery (SPPB).
HIV (*61,62*)	1) The gait speed (GS); 2) Timed-up-and-go test (TUGT); 3) The British Geriatric Society (BGS); 4) The Fried frailty phenotype (FFP); 5) The Short Physical Performance Battery (SPPB); 6) The VACS index; 7) The Clinical Frailty Scale (CFS); 8) Brief Frailty Instrument for Tanzania (B-FIT 2).

debilitation. There are appropriate screening tools for various types of kidney disease. Chronic kidney disease (CKD) is common in older adults. It is associated with frailty and functional limitations and has a heterogenous natural history (*47*). CKD promotes the activation of multiple pro-ageing pathways, which can lead to an early onset of frailty and increase the risks for morbidity and mortality (*48*). Therefore, we need to have early detection of chronic kidney disease through screening tools. For patients with prostate cancer, the Vulnerable Elders Survey-13 (VES-13) questionnaire is very sensitive in the population of older patients with prostate cancer, and therefore, it would be a good frailty screening tool in these patients. Although the VES-13 questionnaire had a large number of false positives, it has a high negative predictive value, which is important statistic for a good screening questionnaire. However, the G8 accurately identifies individuals among those initially detected to be frail using the VES-13 questionnaire (*49*). In addition, for dialysis patients, frailty assessments of incident dialysis patients are moderately to strongly correlated with FI. At the specified FI cutoff values, the overall CFS score (FACT-CFS) and Dialysis Morbidity and Mortality Study (DMMS) are highly sensitive measures of frailty. The CFS and FACT-CFS could be viable alternative screening tools for dialysis patients (*50*).

4.4. Cirrhosis

There is no "gold standard" for the assessment of frailty in cirrhosis. In 2019, the American Society of Transplantation Liver and Intestinal Community of Practice described the tools available for the evaluation of physical frailty in patients with cirrhosis (*51*). These tools included the Short Physical Performance Battery (SPPB), Fried Frailty Criteria (FFC), CFS, Liver Frailty Index (LFI), 6-minute walk test (6MWT), Activities of Daily Living (ADL), cardiopulmonary exercise testing (CPET), gait speed, grip strength, Instrumental Activities of Daily Living (IADL) and Karnofsky Performance Status (KPS). Each tool has its advantages or disadvantages depending upon the setting as the tools vary in the test characteristics, subjectivity, predictive validity for outcomes, reliability, responsiveness to change over time, time taken to administer, and whether specialized equipment or highly trained personnel would be required for testing. For example, the CFS is easy and quick to perform but is subjective. The FFC is lengthy and has some subjective components but is a reliable predictor of outcomes. The LFI is objective but requires specialized equipment. The SPPB is objective without the need for equipment, but includes three tests (similarly to the LFI) and requires more time than a single measure (52). Therefore, when assessing the degree of frailty in patients with cirrhosis, it is important to select the appropriate assessment tool for screening.

4.5. Chronic obstructive pulmonary disease

According to a review, the FFP and FI are the most used tools for assessing frailty in patients with stable chronic obstructive pulmonary disease (COPD), used both in

clinics and rehabilitation centers (*53*). The FFP has been demonstrated to predict mortality and adverse clinical outcomes in community-based patients with stable COPD and hospitalized and immunodeficient patients with advanced COPD (*54*). The SPPB is also a well-established tool for accessing lower limb functional impairment in older adults, and mortality risk in patients with stable COPD (*55*). Zhang *et al*. showed a high prevalence of frailty in older adults with stable COPD assessed using the FFP, CFS, Frail index of accumulative deficits (FI-CD), and the SPPB screening tools Frailty, as assessed by the four assessment tools was associated with poor outcomes, including 1-year acute exacerbation of COPD, hospitalization, or death. The FFP, CFS, FI-CD, and SPPB tools showed comparable performance in predicting 1-year mortality (*56*).

4.6. HIV

People living with HIV are an ageing population with a high prevalence of frailty (*57*). Frailty in people living with HIV has been identified at younger ages than in the general population (*58*), meaning there is a risk of delayed identification of frailty and at a more advanced stage, where some interventions may be less effective, resulting in greater health and social care costs (*59*). The 2019 European AIDS Clinical Society (EACS) guidelines recommended frailty screening in older people living with HIV (*60*). Frailty has increasingly become a cause for concern for caretakers who look after patients with HIV. A study by Beanland *et al*. (*61*) showed that objective measures of frailty screening (gait speed (GS) and timed-up-and-go test) are more closely associated with clinical parameters than the subjective measure of frailty screening done with the self-reported health questionnaire in people with HIV. In another follow-up study of eight years (*62*), frailty and SPPB was significantly associated with increased risk of mortality in middle-aged individuals with HIV. For people living with HIV to gain the most from frailty screening, it is essential that information regarding frailty status is shared in conjunction with a clear plan of the next steps in their care. In addition, services should prioritize the social and psychological aspects of frailty going beyond just the physical domains (*57*).

5. Electronic frailty assessment tool

Primary care screening for frailty status is recommended in clinical guidelines. But is impeded by doctor and nurse workloads and the lack of valid, easy-to-use, and time-saving screening tools (*63*). Vulnerable states can be identified automatically and at scale using electronic screening tools, thereby addressing the current situation of high workload for clinical workers. Over the last few years, COVID-19 pandemic, which is caused by severe acute respiratory distress syndrome coronavirus 2, continues to particularly affect older adults worldwide. The novel coronavirus strain was first detected in December 2019 and the World Health Organization declared the severe acute respiratory distress syndrome coronavirus 2 outbreak a pandemic on March 11, 2020 (*64*). Although the COVID-19 pandemic has disproportionately affected the older populations (*65*), it has been argued that frailty and certain comorbidities, rather than the chronological age, are the main factors influencing the clinical manifestations and pathophysiological mechanisms of COVID-19 (*66*). This makes screening for frailty particularly important. However, since COVID-19 is transmitted by the respiratory tract, an electronic screening tool fits the bill for avoiding exposure. With the continuing global spread of COVID-19, an automated FI could be a useful and efficient tool for risk stratification in hospitalized patients with COVID-19. Based on the deficit accumulation model proposed by Rockwood *et al*. (*28,67*), Clegg *et al*. (*68*) developed and validated an electronic frailty index (e-FI). The e-FI is a 36-item tool with good correlation with hospitalization, longer hospital stays, nursing home admissions, and mortality. The e-FI was associated with in-hospital mortality throughout the pandemic, and it outperformed other frailty and comorbidity measures, including the CFS, the Hospital Frailty Risk Score (HFRS), and Charlson Comorbidity Index (CCI) in discriminating short- and long-term mortality (*69*). However, the e-FI has limited application outside the UK, as the coding system (terms and codes used) is not in the format of the International Classification of Diseases version 10 (ICD-10); hence, it needs to be adapted and validated for transfer to other countries. The e-SIF automatically and instantaneously classifies frailty status in individuals aged ≥ 65 years for whom computerized clinical histories are available (*68*). The e-SIF can predict mortality, hospitalization and institutionalization, and is correlated with health resources consumption. These results suggest that the e-SIF is a valid frailty screening instrument for older adults in a primary care setting. In addition to the e-fi and e-SIF electronic screening tools, the FTA system is equally effective for frailty screening. The FTA system provides results immediately and is an advantageous alternative to traditional manual measurements. The use of the FAT score for predicting pre-frailty will help to provide early interventions to prevent individuals to progress to frailty. The FAT system provides a more convenient and comprehensive frailty screening hence, using this computerized automatic screening platform it might be possible to expand the scope of frailty prevention (*70*).

6. Conclusion

We reviewed global frailty screening tools published between 2001 and 2023. There is currently no gold standard in the Global Frailty Screening tool. Over the

past few decades, frailty screening tools have allowed for early detection and early prevention, but the number of people with debilitating conditions continues to rise globally. Frailty has severe effects on the quality of survival and health outcomes in older adults However, frailty is dynamic and reversible, and can improve or worsen over time. Early identification of frailty in older adults is therefore important for the development of interventions to slow or even reverse the progression of frailty. A summary of the frailty screening tool can help clinical staff to quickly and accurately select a suitable scale, but more work is needed to develop a globally recognized gold-standard scale to screen for frailty. A globally standardized screening tool can allow for earlier effective measures to be taken to improve health problems in older adults, to prevent progression of frailty, and to improve the quality of survival. We intend to conduct a large-scale validation of the frailty screening tools that are available.

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**Address correspondence to:*
Deng Yi, Graduate School of Nursing, Hamamatsu University School of Medicine, Hamamatsu, Shizuoka, Japan.
E-mail: M23012@hama-med.ac.jp

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Review

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The use of artificial intelligence in the treatment of rare diseases: A scoping review

Da He[1,§], Ru Wang[1,§], Zhilin Xu[2], Jiangna Wang[3], Peipei Song[4], Haiyin Wang[1,*], Jinying Su[5,*]

[1] Shanghai Health Development Research Center (Shanghai Medical Information Center), Shanghai, China;
[2] EYE & ENT Hospital of Fudan University, Shanghai, China;
[3] Jiangxi University of Chinese Medicine, Shanghai, China;
[4] Center for Clinical Sciences, National Center for Global Health and Medicine, Tokyo, Japan;
[5] Shanghai University of Traditional Chinese Medicine, Shanghai, China.

SUMMARY With the increasing application of artificial intelligence (AI) in medicine and healthcare, AI technologies have the potential to improve the diagnosis, treatment, and prognosis of rare diseases. Presently, existing research predominantly focuses on the areas of diagnosis and prognosis, with relatively fewer studies dedicated to the domain of treatment. The purpose of this review is to systematically analyze the existing literature on the application of AI in the treatment of rare diseases. We searched three databases for related studies, and established criteria for the selection of retrieved articles. From the 407 unique articles identified across the three databases, 13 articles from 8 countries were selected, which investigated 10 different rare diseases. The most frequently studied rare disease group was rare neurologic diseases (n = 5/13, 38.46%). Among the four identified therapeutic domains, 7 articles (53.85%) focused on drug research, with 5 specifically focused on drug discovery (drug repurposing, the discovery of drug targets and small-molecule inhibitors), 1 on pre-clinical studies (drug interactions), and 1 on clinical studies (information strength assessment of clinical parameters). Across the selected 13 articles, we identified total 32 different algorithms, with random forest (RF) being the most commonly used (n = 4/32, 12.50%). The predominant purpose of AI in the treatment of rare diseases in these articles was to enhance the performance of analytical tasks (53.33%). The most common data source was database data (35.29%), with 5 of these studies being in the field of drug research, utilizing classic databases such as RCSB, PDB and NCBI. Additionally, 47.37% of the articles highlighted the existing challenge of data scarcity or small sample sizes.

Keywords artificial intelligence, rare diseases, treatment

1. Introduction

Rare diseases are defined as illnesses with a low incidence rate. Different countries and regions have varying specifications for the incidence rates of rare diseases. In the European Union, diseases with an incidence rate lower than 1 in 2,000 are considered rare diseases (*1*); in the United States, rare diseases are defined as those affecting fewer than 200,000 individuals annually (or an incidence rate less than 1 in 1,500) (*2*); in Japan, rare diseases are specified as those affecting fewer than 50,000 individuals (or an incidence rate of 1 in 2,500) (*3,4*). According to the World Health Organization (WHO), rare diseases are those where the number of individuals affected constitutes 0.65‰ to 1‰ of the total population (*5*). With the continuous advancement in disease diagnostic technologies, the increasing subdivision in the field of diseases, and the yearly improvement in data statistics, new diseases have been continually identified or included since the definition of rare diseases was established, leading to an increase in the types of rare diseases. There are currently more than 7,000 known rare diseases globally (*6*), with an estimated accumulated prevalence of 3.5–5.9% and affecting more than 400 million people worldwide (*7,8*).

Due to the difficulty in diagnosing rare diseases, many are treated as common illnesses or remain undetected, suggesting that the actual number of patients is likely higher than statistical estimates. Furthermore, with advancements in diagnostic technologies and increased health literacy among populations, the number of individuals identified with rare diseases continues to expand (*9*).

Currently, due to unclear etiologies, the small number

of patients, among other reasons, rare disease patients face significant challenges compared to those with common diseases such as hypertension and diabetes. These challenges include difficulties in diagnosis, a lack of specific treatment techniques post-diagnosis, or the inability to afford available medications (*10,11*). Globally, only about 5% of rare diseases have effective treatment methods available. Even for rare diseases with existing treatment options, the cost of medications is often prohibitively expensive, imposing a substantial economic burden on both patients and society (*12*).

In recent years, due to the large volume and structural nature of data in the medical field, which align well with the needs of artificial intelligence, the development and application of artificial intelligence (AI) has permeated various domains within healthcare (*13-18*). In the field of rare diseases, the majority of AI applications pertain to disease screening, diagnosis, and prognosis, areas in which there is a substantial body of related literature (*19-21*), and where comprehensive review studies have already categorized and summarized the findings. While screening, diagnosis, and prognosis are undoubtedly important, for the vast number of patients with confirmed rare diseases, the need for treatment is more urgent (*22*). Currently, research and applied studies of AI in the treatment of rare diseases are relatively scarce, and there are no comprehensive review studies on the use of AI in the treatment of rare diseases.

In this scoping review, we explore scientific literature to investigate the application of AI in the treatment of rare diseases, and identify the key features used to train these AI models based on the pursued objectives. This includes specific groups of rare diseases that have been highlighted, therapeutic areas, key algorithms employed, data types used in models, as well as potential opportunities and challenges. These functionalities can support the future development of AI in the treatment of rare diseases.

2. The scoping review methods

This scoping review was followed to be designed and performed by the Preferred Reporting Items for Systematic reviews and meta-Analyses extension for scoping reviews (PRISMA-ScR) guideline (*23*).

2.1. Literature search

We conducted a systematic search across three databases for eligible articles: *i*) PubMed, *ii*) Web of Science, and *iii*) the Institute of Electrical and Electronics Engineers (IEEE) Xplore. The specific search terms used are detailed in Table 1 (Online Data, *http://www.irdrjournal.com/action/getSupplementalData.php?ID=179*).

Within the PubMed database, our primary search terms were "Rare Diseases/therapy"(Mesh) and "Artificial Intelligence"(Mesh). To comprehensively retrieve articles in the domain of artificial intelligence, we also included all associated entry terms related to artificial intelligence in PubMed. In addition, in order to extensively search for applications of rare diseases in the therapeutic area, we reviewed all 26 MeSH terms associated with the term "therapy" in the MeSH Database of PubMed. Based on this, we identified six terms – virtual reality exposure therapy, computer assisted therapy, radiotherapy, wearables, surgical robot, and drug therapy – as interventions for rare diseases. These terms were then combined with "Rare Diseases"(Mesh) for filtration.

In the IEEE Xplore, our search terms were ("All Metadata": Rare Diseases) combined with the aforementioned six therapeutic interventions. This combined search was further merged with the term ("All Metadata": artificial intelligence).

In the Web of Science database, our search terms were TS = (Rare Diseases) OR TS = (orphan disease), which were then combined with the six treatment methods. This combined search was further merged with the term TS = (Artificial Intelligence). Additionally, we incorporated the search terms "machine learning" and "deep learning".

2.2. Literature selection

After obtaining the potential articles, we conducted a screening of abstracts and full texts. The following criteria were employed to identify relevant literature concerning the use of artificial intelligence methods for rare disease treatment: *i*) Written in English, *ii*) Published or publicly available (*e.g.* conference proceedings) between January 1, 2010, and August 31, 2023. And exclusion criteria included: *i*) Not published in peer-reviewed journals or conference proceedings (*e.g.* preprints), *ii*) Not original research (*e.g.* reviews, editorials), *iii*) Not human patient data or scientific texts or publications (*i.e.*, articles using animal or simulated data were excluded), and *iv*) not rare disease topic.

2.3. Data extraction and synthesis

One reviewer (WJN) developed and extracted metadata for each article. Two reviewers (WR and HD) verified and refined the metadata for completeness. Upon selecting the relevant studies based on the eligibility criteria, the metadata extracted from these articles included: *i*) Publication year; *ii*) Country where the study was conducted (according to the senior author's affiliation); *iii*) Rare disease (diseases were specified using the Orphanet disorder name); *iv*) Rare disease group (according to the "preferential parent" of the disease as defined in the hierarchy of the Orphanet classification (*24*), the classification is based on the 34 disorder groups updated on Orphant's website in July 2023 (*25*); *v*) Therapeutic area; *vi*) Treatment method

(interventions used for rare diseases); *vii*) Purpose of using AI (objective of the study); *viii*) AI architecture (including types of algorithms such as deep learning and machine learning); *ix*) Data type; and *x*) Challenges. Table 2 (Online Data, *http://www.irdrjournal.com/action/getSupplementalData.php?ID=179*) summarizes the metadata elements.

3. Specific status description

The literature search identified a total of 407 unique records. After screening and assessing the articles for eligibility, 13 articles were included in the final analysis (The list of the selected 13 articles with their metadata elements extracted is available in Supplementary Table S1, *http://www.irdrjournal.com/action/getSupplementalData.php?ID=180*). Figure 1 displays the Preferred Reporting Items for Systematic reviews and meta-Analyses (PRISMA) flow diagram for article selection. After de-duplication, the literature search identified a total of 407 unique records, 281 articles were included in full-text screening. After screening and assessing the articles for eligibility, 13 articles were included in the final analysis.

3.1. Yearly publication trend

These studies originated from eight different countries. The majority of the publications (n = 5, 38.46%) came from the United States, followed by Italy (n = 2, 15.38%); Canada, Austria, France, India, Japan, and China each contributed one publication (n = 1, 7.69%).

Over the 13-year span from 2010 to 2023, articles meeting the criteria were found from 2017 to 2022. The number of articles rose from 1 in 2017 to 5 in 2021, and four articles were identified in 2022. Since the beginning of 2023 and as of August 31, 2023, no articles have been published that fulfill the selection criteria concerning the use of AI in the treatment of rare diseases.

3.2. Rare diseases and rare diseases group

Ten unique rare diseases were identified from the reviewed articles, with 3 articles focusing on general rare diseases rather than specific conditions. Table 3 presents the rare diseases identified in the selected articles, along with their corresponding orpha numbers. We classified the rare diseases identified from the reviewed articles into rare disease groups using the hierarchical structure defined by Orphanet. If an article aims to investigate specific characteristics of rare diseases that can be applied to general rare diseases. A rare disease can qualify for multiple groups, and the group as defined by the preferential parent in the classification hierarchy.

Of the 10 diseases, 3 (30%) had a prevalence of 1-9/100,000 patients, 2 (20%) had a prevalence of 1-9/1,000,000, 2 (20%) had a prevalence of 1–5/10,000, and 1 (10%) < 1/1,000,000, and for the remaining two diseases, Orphanet did not provide their prevalence.

The groups of rare diseases identified are illustrated in Figure 2 (Online Data, *http://www.irdrjournal.com/action/getSupplementalData.php?ID=179*). The most frequent group was rare neurologic disease, accounting for 5 out of 13 articles (38.46%). All other groups were represented by just one article each.

3.3. Therapeutic area and method

As illustrated in Figure 3 (Online Data, *http://www.irdrjournal.com/action/getSupplementalData.php?ID=179*), from the 13 articles selected based on our criteria, we identified four therapeutic areas. Among

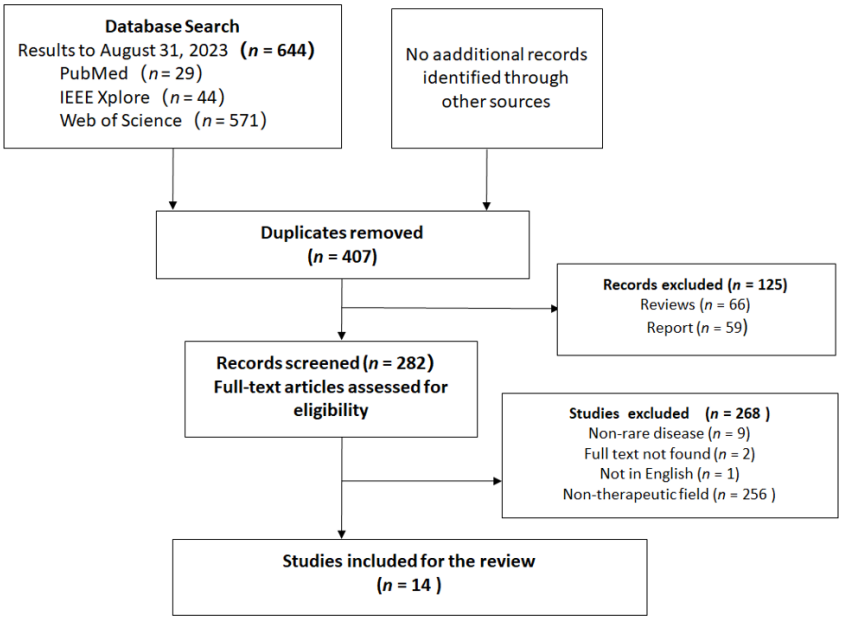

Figure 1. PRISMA flow diagram for literature selection.

Table 3. Rare diseases included in the selected literature

Rare disease	Orpha number (ORPHA)	Prevalence	Rare disease group (preferential parent)	Number of studies	Use cases (*Ref*)
General rare disease	NA	none	NA	3 (23.08%)	*26-28*
Metachromatic leukodystrophy	512	1–9/1,000,000	Rare neurologic disease	1 (7.69%)	*29*
Glycogen storage disease due to acid maltase deficiency	365	1–9/100,000	Rare genetic disease	1 (7.69%)	*30*
Canavan disease	141	Unknown	Rare neurologic disease	1 (7.69%)	*31*
Creutzfeldt-Jakob	204	< 1/1,000,000	Rare neurologic disease	1 (7.69%)	*32*
Pleural mesothelioma	50251	1–9/100,000	Rare neoplastic disease	1 (7.69%)	*33*
Alkaptonuria	56	1–9/1,000,000	Rare inborn errors of metabolism	1 (7.69%)	*34*
Acute Myeloid Leukaemi	519	1–5/10,000	Rare hematologic disease	1 (7.69%)	*35*
Congenital cataract microcornea with corneal opacity	289499	Unknown	Rare ophthalmic disorder	1 (7.69%)	*36*
Amyotrophic Lateral Sclerosis	803	1–9/100,000	Rare neurologic disease	1 (7.69%)	*37*
Canavan disease	141	Unknown	Rare neurologic disease	1 (7.69%)	*38*

these, 7 articles (53.85%) focused on drug research, 3 articles (23.08%) on precision medicine, 2 articles (15.38%) on health management, and 1 article (7.69%) on personalized services.

It's noteworthy that, of the 7 articles in the drug research area, 5 were focused on the area of drug discovery (*e.g.*, drug repurposing, the discovery of small-molecule drugs), 1 on pre-clinical studies (drug interactions), and 1 on clinical studies (information strength assessment of clinical parameters).

3.3.1. Drug research

Specifically, among the 5 articles focusing on drug discovery, 3 focused on the field of drug repurposing (*27-29*), that is, linking disease mechanisms with drug effects. Drug repurposing plays a significant role in the field of drug discovery. Foksinska *et al.* mentioned that identifying treatments for rare diseases is challenging due to limited understanding of disease mechanisms, small cohort sizes, inter-individual symptom variability, and little commercial incentive to develop new treatments. One promising therapeutic avenue was drug repurposing, where FDA-approved drugs were repurposed as new treatments (*26*). Challa *et al.* asserted that in its current state, ML (machine learning) was best leveraged in the field of drug repurposing to inform human "go/no-go" decision-making (*39*). Table 4 displays the overview of articles of drug research.

Cong *et al.* proposed a novel two-stage prediction approach for drug repurposing based on machine learning. This methodology clustered diseases based on gene expression patterns and evaluates drug efficacy through the reversibility of abnormal gene expression. It identified 22 drugs such as KM 00927, I-BET, alvocidib, and vorinostat, as candidates for repurposing, which had high efficacy against specific diseases like inclusion body myositis, polymyositis, and dermatomyositis (*27*). Sosa *et al.* proposed a literature-based knowledge graph embedding method for identifying drug repurposing opportunities in rare diseases. The method leveraged a large knowledge graph, the Global Network of Biomedical Relationships (GNBR), which integrated information from pharmacology, genetics, and pathology to generate drug repurposing hypotheses. The method achieved high performance on a gold-standard test set of known drug indications (AUROC = 0.89) and is capable of generating hypotheses for novel applications of existing drugs (*28*). Esmail *et al.* constructed an artificially induced whole-brain organoid platform (NEUBorg), which serves as an advanced iteration of the previously validated machine learning platform, DeepNEU (v6.2). Using NEUBorg, they generated artificially induced whole-brain organoids (aiWBO) simulations of metachromatic leukodystrophy (MLD) and provided a new method to evaluate factors related to MLD pathogenesis, disease progression and new potential treatment options. Utilizing this method, the authors identified 861 single and dual drug combinations as potential therapeutic targets for MLD. The study comprehensively summarizes the drug repurposing outcomes and the pharmaceuticals evaluated in the simulations (*29*).

One study focused on the discovery of drug targets. Esmail *et al.* constructed an AI platform, DeepNEU v3.6, to support target identification for Infantile Onset Pompe Disease (IOPD). This platform is capable of generating computer-simulated stem cells (aiPSC) and differentiated skeletal muscle cells (aiSkMC) models, both with and without the expression of Acid Alpha-Glucosidase (GAA). These simulations were validated using peer-reviewed results from existing literature to assess calcium homeostasis and mitochondrial function in IOPD patients. The authors employed aiSkMC IOPD simulations to identify known and novel biomarkers as well as potential therapeutic targets. Ultimately, the aiSkMC model for IOPD accurately predicted gene and phenotypic features reported in recent literature (*30*).

Another study focuses on the discovery of lead compounds for small-molecule inhibitors. Stecula *et al.* introduced a deep convolutional neural network, AtomNet, to identify lead compounds that inhibit Aspartate N-Acetyltransferase (ANAT), targeting the treatment of Canavan disease. The authors demonstrate AtomNet's capability to identify novel active scaffolds under challenging constraints, such as the scarcity or complete unavailability of target data, and thereby supporting early-stage drug discovery efforts (*31*).

Table 4. Overview of articles of drug research

Therapeutic area	Sub-area	Method	Algorithm	Product	Function and Result	*Ref.*
Drug research	Drug discovery	Drug repurposing	k-means, UMAP	A two-stage prediction approach for drug repurposing	This approach identified 22 drugs such as KM 00927, I-BET, alvocidib, and vorinostat, as candidates for repurposing, which had high efficacy against specific diseases like inclusion body myositis, polymyositis, dermatomyositis.	*27*
			UKG, UMAP	A literature-based knowledge graph embedding method	The method achieving high performance on a gold-standard test set of known drug indications (AUROC = 0.89) and is capable of generating hypotheses for novel applications of existing drugs.	*28*
			RNN, CM, SVM, GA	NEUBOrg	Could generate aiWBO simulations of MLD to evaluate Factors related to MLD pathogenesis, disease progression and new potential treatment options. The authors identified 861 single and dual drug combinations as potential therapeutic targets for MLD.	*29*
		Discovery of drug targets	RNN, CM, SVM, GA	DeepNEU v3.6	This platform is capable of generating aiPSC and aiSkMC models, both with and without the expression of GAA. The aiSkMC model for IOPD accurately predicted gene and phenotypic features reported in recent literature.	*30*
		Discovery of lead compounds for small-molecule inhibitors	CNN	AtomNet	to identify lead compounds that inhibit ANAT, targeting the treatment of Canavan disease.	*31*
	Pre-clinical research		LR, KNN, SVM, DT, RF, XGBoost	A model for studying the relationship between drugs used in the treatment of CJD and their impact on clinical parameters.	This model could suggest appropriate drugs upon the input of clinical parameters. The results indicated that Random Forest outperformed XGBoost, with an average accuracy of 98.39%.	*32*
	Clinical research		RF	Highlighted the informative power of clinical parameters in predicting initial response to treatment.	To predict the initial treatment response of patients with advanced/unresectable pleural mesothelioma and evaluate different clinical parameters (including gender, tissue type, BMI, smoking habits, number of packs/year and disease stage, etc.) and the correlation with response. The average AUC value of the model was 77.0%, the accuracy was 75%, the sensitivity was 74.8%, and the specificity was 83.3%.	*33*

In addition, one study focuses on pre-clinical research concerning drug interactions. Rajagopal *et al.* employed machine learning algorithms to develop a model for studying the relationship between drugs used in the treatment of Creutzfeldt-Jakob Disease (CJD) and their impact on clinical parameters. This model could suggest appropriate drugs upon the input of clinical parameters. The study evaluated various machine learning algorithms, such as Logistic Regression (LR), K-Nearest Neighbor (KNN), Decision Tree Classifier(DT), Support Vector Machine (SVM), Exterme Gradient Boosting (XGBoost), and RF. The results indicated that RF outperformed XGBoost, with an average accuracy of 98.39% (*32*).

Furthermore, one study highlighted the informative power of clinical parameters in predicting initial response to treatment. Massafra *et al.* used the RF algorithm combined with the sequential forward feature selection procedure to predict the initial treatment response of patients with advanced/unresectable pleural mesothelioma and evaluated different clinical parameters (including gender, tissue type, BMI, smoking habits, number of packs/year and disease stage, *etc.*) and the correlation with response. The average AUC (Area Under Curve) value of the model was 77.0%, the accuracy was 75%, the sensitivity was 74.8%, and the specificity was 83.3% (*33*).

3.3.2. Precision medicine

Drug repurposing can be difficult and requires depth of knowledge across multiple fields, which is complicated by the rapid pace of biomedical knowledge discovery.

To address these challenges, Foksinska *et al.* developed MediKanren, an artificial intelligence tool that used mechanistic insights into genetic diseases to identify treatment options to enable precision medicine for rare diseases. Utilizing knowledge graphs, mediKanren could effectively link all relevant literature and databases. The tool enabled a scalable process that has been used to help more than 500 rare disease families *(26*). Spiga *et al*. used machine learning algorithms, especially the RF algorithm, to establish a comprehensive digital ecosystem, AreciseKUre, which collected and analyzed data on the rare genetic disease Alkaptonuria (AKU), a digital platform aimed at creating a Precision Medicine Ecosystem (PME). AreciseKUre could determine the most suitable treatment method for AKU patients based on their quality of life (QoL) scores before and after medication. It represented a proof of principle study that could be applied to other rare diseases, allowing data management, analysis and interpretation (*27*). Licandro *et al.* proposed a novel method, WGAN-NN, for the computational quantification of Acute Myeloid Leukemia (AML) cancer cells in blood samples. WGAN-NN is a semi-supervised learning approach that embeds a Fully Connected Neural Network (FNN) within a Wasserstein Generative Adversarial Network (WGAN). The results show that the proposed semi-supervised WGAN embeddings are superior to PCA-NN and FNN (*35*). Table 5 displays the overview of articles of precision medicine.

3.3.3. Health management and personalized service

Long *et al.* introduced an artificial intelligence agent (AI agent), CC-Cruiser, that used deep learning for the diagnosis, risk stratification, and therapeutic recommendations for congenital cataracts. The AI agent was integrated with a cloud-based, multi-hospital collaboration platform aiming to improve disease management for patients with rare diseases such as congenital cataracts. Results indicated that CC-Cruiser accurately diagnosed congenital cataracts and provided treatment decisions across computational tests, web-based studies, needle-in-a-haystack tests, and multi-hospital clinical trials. The authors also demonstrated that the performance of the AI agent was comparable to that of individual ophthalmologists (*36*). Kmetzsch *et al.* presented a novel disease progression score using cross-sectional multimodal data to assess frontotemporal dementia and amyotrophic lateral sclerosis, a rare neurodegenerative disease. This framework leveraged neuroimaging and microRNA data to train supervised multimodal variational autoencoders to learn meaningful latent spaces that represented disease progression (*37*).

Chapron *et al.* discussed the impact of myotonic dystrophy type 1 (DM1) on the quality of life and daily activities of affected individuals in Quebec, Canada. To mitigate the effects of DM1, the authors proposed a complete assistance system that provided training guidance and motivation, Acti-DM1, which identified movement-related activities and monitored each exercise performed during training sessions (*38*). Table 6 displays the overview of articles of health management and personalized service.

3.4. Purpose of using AI and algorithms

In the 13 articles, we identified a total of 20 different algorithms (multiple algorithms were often studied in a single article. One article did not specify a particular algorithm but utilized the AI platform medKanren, which we categorized as "other"). The total count of algorithms was 32. The most frequently used algorithm was RF, accounting for 4 instances (12.50%); followed by SVM with 3 instances (9.37%); Subsequent to these were DT, KNN, Uniform Manifold Approximation and Projection (UMAP), Generative evolutionary algorithms (GA), Confusion Matrix (CM), Recurrent Neural Network (RNN), and Convolutional Neural Networks (CNN), each with 2 instances (6.25%); The algorithms k-means, WGAN, LR, XGBoost, FNN, Uncertain Knowledge Graph (UKG), Multilayer Perceptron (MLP), Naive Bayes (NB), Principal Component Analysis (PCA), Variational Autoencoder (VAE); and "Other" accounted for 1 instance (3.13%) (Figure 4, Online Data, *http://www.irdrjournal.com/action/getSupplementalData.php?ID=179*).

In the 13 articles, we identified 15 purposes for the use of AI in rare disease treatment, categorized into 4 types (a single article might have more than one

Table 5. Overview of articles of precision medicine

Therapeutic area	Algorithm	Product	Function and Result	(*Ref*)
Precision medicine	Other	MediKanren	MediKanren could effectively link all relevant literature and databases. The tool enabled a scalable process that has been used to help more than 500 rare disease families	*26*
	RF	AreciseKUre	AreciseKUre could determine the most suitable treatment method for AKU patients based on their quality of life (QoL) scores before and after medication.	*34*
	WGA, PCA, FNN	WGAN-NN	To calculate the number of AML cancer cells in blood samples. The results show that the proposed semi-supervised WGAN embeddings are superior to PCA-NN and FNN.	*35*

Table 6. Overview of articles of health management and personalized service

Therapeutic area	Algorithm	Product	Function and Result	(*Ref*)
Health management	CNN	CC-Cruiser	CC-Cruiser accurately diagnosed congenital cataracts and provided treatment decisions across computational tests, web-based studies, needle-in-a-haystack tests, and multi-hospital clinical trials. The authors also demonstrated that the performance of the AI agent was comparable to that of individual ophthalmologists.	*36*
	VAE	A novel disease progression score using cross-sectional multimodal data to assess frontotemporal dementia and amyotrophic lateral sclerosis.	This framework leveraged neuroimaging and microRNA data to train supervised multimodal variational autoencoders to learn meaningful latent spaces that represented disease progression.	*37*
Personalized service	RF, DT, SVM, KNN, NB, MLP	Acti-DM1	The system could identify movement-related activities and monitored each exercise performed during training sessions.	*38*

objective). The most prevalent purpose was to improve the performance of analytical tasks (*e.g.* improving model performance), accounting for 9 articles (53.33%); Proof-of-concept (*e.g.* analyzing the impact of drugs on clinical parameters) accounting for 3 articles (20.00%); To address the challenge of data scarcity was the goal in 3 articles (18.75%); and to reduce manual effort (*e.g.* leveraging AI tools for virtual drug screening to diminish the time and cost of manual experimentation) was highlighted in 1 article (6.67%) (Figure 5, Online Data, *http://www.irdrjournal.com/action/getSupplementalData.php?ID=179*).

3.5. Data type

Over the 13 articles, we identified a total of 7 distinct data sources, cumulatively accounting for 18 data points (Note that each paper might reference more than one data type). The predominant source of data was derived from databases, represented in 6 articles (35.29%). Following this, image data, exemplified by CT scan datasets, was cited in 3 articles (17.65%). Omics data (*e.g.* genomics, proteomics), demographic data (*e.g.* age), and functional test data (*e.g.* pulse, blood pressure, and other physiological metrics) were each represented in 2 articles (11.76%). Both literature-derived data and experimentally obtained data were cited in a single article each, contributing to 5.88% of the data sources.

It is noteworthy that within the data sourced from databases, 5 articles pertained specifically to the domain of drug development, with databases such as RCSB PDB and NCBI – quintessential repositories in the drug development landscape – being employed. Meanwhile, 2 articles employed database-derived data in the context of precision therapy (*e.g.*, ApreciseKUre) (Figure 6, Online Data, *http://www.irdrjournal.com/action/getSupplementalData.php?ID=179*).

3.6. Challenges in using the treatment of AI for rare diseases

We classified the challenges in using deep learning for rare disease research reported by the authors into 8 categories (Note that each paper might reference more than one challenge, and if no challenges were highlighted, they were labeled as "None"). Among the identified 19 challenges in applying AI in the field of rare diseases treatment, 10 articles (47.37%) mentioned the primary challenge is lack of sufficient data, indicating that the amount of available data has become the foremost challenge in using AI for rare disease research.

Enhancing interpretability stands as an imperative when applying AI in healthcare and medicine (*40,41*). While a multitude of studies have achieved commendable performance in their target analysis tasks, certain limitations warrant attention. Specifically, 15.76% (n = 3) of the articles stated that there's a need to have models with better interpretability. Additionally, 10.53% (n = 2) of the papers highlight the constrained availability of model options, indicating a pertinent need for diversified algorithms. The necessity for wet-lab validation within the realm of pharmaceutical research, signifies that future endeavors must bolster the reliability of AI methodologies. Another article highlighted that computational capabilities of wrist-worn devices used in their study were limited, necessitating a reduction in feature dimensions for machine learning algorithms (*38*). One article articulated that the dearth of suitable therapeutic options and limited acquired knowledge could potentially inhibit the utility of AI in the domain of rare diseases (*26*) (Figure 7, Online Data, *http://www.irdrjournal.com/action/getSupplementalData.php?ID=179*).

4. The status quo and problems of AI application in the treatment of rare diseases

AI has been extensively utilized in various types of fundamental research to date. With the advent and rapid iteration of large language models such as ChatGPT, AI's involvement has emerged in the therapeutic domains of

many diseases (*42-46*). This study provides a review of research applying AI technologies in the treatment of rare diseases, aiming to understand the current global contributions of AI in the field of rare disease therapy.

4.1. The application of AI in the treatment of rare diseases emerged later and is relatively scarce

Although the concepts of rare diseases and artificial intelligence were introduced quite some time ago, research on the application of AI in the treatment of rare diseases did not emerge until 2017. Over time, the number of studies has not seen a substantial increase, unlike in the areas of rare disease prevention, screening, and prognosis (*47,48*).

In terms of the countries involved in the research, the United States leads in applying AI to the treatment of rare diseases, followed by Italy, Canada, Australia, and France – countries that are more advanced in biomedicine and artificial intelligence – as well as three Asian countries: Japan, China, and India. However, overall, the number of studies on the application of AI in the treatment of rare diseases is relatively low across all countries.

From the perspective of the disease categories included in the literature, a relatively higher proportion of studies focuses on neurological aspects, suggesting that research on neurological drugs and therapies is more prevalent among various rare disease treatments.

The complete lifecycle of a disease includes prevention, screening, diagnosis, treatment, and rehabilitation. In this process, compared to diagnosis, screening, and prognosis of rare diseases, studies on the application of AI in their treatment are noticeably less. In a 2020 scoping review on the application of machine learning in the field of rare diseases, of the 211 included studies, 40.8% used machine learning for diagnosis, 38.4% for prognosis, and only 4.7% for treatment (*48*). A systematic review included studies on the use of machine learning for diagnosis and prognosis, published in July 2023, and 22 studies were included (*19*). The timeframe for literature retrieval in this study was similar to that of our research, yet the number of studies incorporated was 1.6 times greater than those included in our study.

Of the 13 articles included in this study, more than half aimed to improve the performance of analytical models, while only 3 addressed the significant data scarcity issue in rare diseases. This highlights the considerable challenges in applying AI technology to treatment of rare diseases. Besides, prevention and screening of rare diseases aim for earlier and faster treatment of symptoms. For the large number of patients already diagnosed with rare diseases and for those who cannot avoid being born with rare conditions in the future, the need for effective treatment is more urgent than prevention and screening. Therefore, it is recommended that global efforts further prioritize research on rare disease medications and treatment methods, fully leveraging advanced algorithms and models such as AI to accelerate the development of corresponding therapies.

4.2. Drug research is the primary domain for AI application in the treatment of rare diseases

In the field of innovative drug research, the 'Double Ten Law' - indicating that it takes 10 years and an investment of one billion US dollars to develop a single innovative drug - is widely recognized globally due to the long development cycle, high investment, and substantial risk associated with drug research (*49,50*). For rare diseases, the development of innovative drugs is even more challenging due to their low incidence rates, complex pathogenic mechanisms, insufficient numbers of subjects for clinical trials, and lower expected product sales, all of which reduce the commercial incentive for drug development compared to common diseases (*51-54*).

The role of artificial intelligence in drug development primarily includes: First, target identification – AI can analyze large-scale data, such as genomic and proteomic data, to identify potential drug targets and predict which targets have a higher likelihood of success. Second, compound screening – following target identification, AI can be used to screen large databases to find compounds that may interact with the target and predict which compounds are more likely to be successful, allowing researchers to prioritize these molecules for further testing. Third, compound optimization – AI can also be used to optimize the chemical structure of compounds, predicting how changes in chemical structure can affect the properties of the compound, such as its ability to bind to the target and potential toxicity, thus enhancing its efficacy and safety. Fourth, clinical trial design – AI can analyze past trial data and predict which patients are most likely to benefit from a specific drug, thereby conducting more effective clinical trials. Fifth, AI can help scientists more quickly hypothesize and validate potential treatments for rare diseases (*55-57*).

According to the review results, one of the main strategies to address the challenges in drug development for rare diseases is repurposing of existing drugs, where AI algorithms, including deep learning and knowledge graph technologies, can play a significant role. By deepening the understanding of drug targets and compound synthesis through AI, existing drugs can be repurposed for new indications or used in combination to treat rare diseases. Additionally, using machine learning, deep learning, and other intelligent algorithms to predict potential gene targets for rare diseases, accurately identify possible rare disease drugs, and suggest appropriate medications for different patients are effective methods to address the challenges in drug development for rare diseases (*58*).

With the assistance of AI, the research time for therapeutic drugs for rare diseases can be significantly reduced, and the efficiency of development greatly enhanced, thereby lowering costs and increasing the likelihood of successful drug research.

4.3. AI contributes to enhancing the efficiency of precision treatment and health management for patients with rare diseases

Based on the understanding of some pathogenic genes responsible for rare diseases, AI can be utilized for its powerful capability in linking, expanding, learning, and computing large datasets. By integrating algorithms with specific databases, it is possible to conduct exploratory drug pairing or testing at the genetic and molecular level, thereby precisely identifying treatment methods for rare diseases. Additionally, AI can identify subgroups of patients most likely to respond to specific treatments, thereby increasing the effectiveness of therapy and reducing the risk of adverse events. Furthermore, AI can circumvent the inefficient resource allocation inherent in traditional human-led health management, widely disseminating the expertise of highly skilled physicians and enabling long-term health management of patients (*59*).

4.4. The lack of high-quality data and the difficulty in constructing and interpreting complex models are the primary challenges in applying AI to rare disease treatment

Although AI demonstrates immense potential in drug repurposing, precision treatment, health management, and personalized services for rare diseases, numerous challenges need to be addressed before its full potential can be realized, given its relatively recent emergence.

First, the lack of high-quality data presents a significant challenge. AI algorithms depend on large volumes of high-quality data for learning and making accurate predictions. However, the low incidence of rare diseases results in a smaller data pool, which can impact the development of AI algorithms. Additionally, the quality of this limited data is subject to various factors, potentially limiting the effectiveness of AI algorithms. In drug discovery, this implies the necessity of acquiring more high-quality data regarding drug targets, chemical structures, and biological pathways.

Second, the complexity in model construction and interpretation poses a challenge. The high complexity of biological systems necessitates the development of algorithms that can predict interactions between drugs and biological systems and generate novel candidate drugs that are both safe and effective. While there are existing methods to extract feature importance from black-box models or to visualize some operational mechanisms of models, comprehensively understanding and interpreting the inner workings and reasoning processes of complex models remain significant challenges.

4.5. Ethical considerations in the use of AI for rare disease treatment

The application of AI in drug discovery also faces ethical and regulatory challenges, including ensuring the safety and efficacy of drugs designed by AI and avoiding unintended consequences. Furthermore, it is crucial to ensure that the use of AI is responsible and adheres to ethical standards (*60-62*).

4.6. Limitations

There are some limitations to our study. Primarily, the scope of investigation was restricted to research implementing artificial intelligence methodologies within the domain of rare disease treatment. Despite the paucity of publications on this specific subject, articles were not excluded based on the caliber of their publishing medium (*e.g.* Journal Impact Factor, JIF), potentially introducing a degree of bias into the review outcomes. Furthermore, the exhaustive list of 7,000+ rare disease instances reported by Orphanet was not entirely leveraged as search terms, potentially precluding the identification of pertinent articles on a broader spectrum of rare diseases. A final constraining factor was the exclusive consideration of articles penned in English, possibly omitting germane articles written in other languages.

5. Conclusion

First, the treatment of rare diseases is equally as important as prevention and screening. However, current AI research in rare diseases is disproportionately focused on drug development, with less emphasis on treatment. There is a need for continued research in drug repurposing, target discovery, hit compound identification, precision treatment, health management, and personalized services. Second, the development and interpretability of algorithms remain one of the main barriers in the treatment of rare diseases. There is potential for the application of large language models in the treatment of rare diseases. Last, to address the issue of insufficient data for rare disease treatment, it is worth considering the expansion of data sources beyond existing databases to include real-world data, especially the establishment of global databases for individual diseases.

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[§]These authors contributed equally to this work.
**Address correspondence to:*
Haiyin Wang, Shanghai Health Development Research Center (Shanghai Medical Information Center), No. 181, Xinbei Road, Minhang District, Shanghai 201199, China.
E-mail: wanghaiyin@shdrc.org

Jinying Su, Shanghai University of Traditional Chinese Medicine, No.1200 Cailun Road, Zhangjiang Hi-Tech Park, Pudong New Area, Shanghai 201203, China.
E-mail: szysujinying@163.com

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Review

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Situs viscerum inversus and abdominal aortic aneurysm: A systematic review of a rare association

Paolo Ossola[1,*], Federico Mascioli[2], Andrea Pierre Luzzi[3], Lorenzo Epis[4], Diego Coletta[5,6]

[1] Department of Surgery, Cardinal Massaia Hospital, Corso Dante Alighieri, Asti, Italy;
[2] Department of Surgery, Casa di Cura Privata Pierangeli Srl, Pescara, Italy;
[3] Department of Medicine DIMI, University of Genova, Genova, Italy;
[4] Department of Surgical Sciences DISC, University of Genova, Genova, Italy;
[5] Department of Surgical Sciences, Policlinico Umberto I University Hospital, Sapienza University of Rome, Rome, Italy;
[6] Hepatopancreatobiliary Surgery, IRCCS Regina Elena National Cancer Institute, Rome, Italy.

SUMMARY Situs viscerum inversus (SVI) is a very rare condition in that abdominal and thoracic organs are located reversed. Abdominal aortic aneurysm (AAA) is a life-threatening pathology due to progressive aortic enlargement until the rupture. The association between SVI and AAA is very infrequent. The aim of this study is to identify the surgical procedures available to treat AAA in SVI. We performed a literature review of all studies about AAA in SVI patients, analyzing PubMed/MEDLINE, EMBASE, Web of Science (WOS), Google Scholar databases. The survey includes all publications until June 2023. The outcomes include demographic findings, type of surgical procedure, intraoperative and postoperative complications, follow-up. A total of 12 studies, including 12 patients, were considered eligible for the review. AAA mean size was 70.5 mm (range: 55–90 mm); the most common localization was in the infrarenal aortic portion. 6 studies reported data on elective surgery, and 6 on emergency procedures. In one case endovascular treatment was performed. No intraoperative complications are reported; 3 postoperative complications are registered. Medium follow-up period was 13.5 months (range: 3–60). According to the available literature, the treatment of AAA in SVI is feasible and does not show an incremented morbidity compared to patients with a normal visceral configuration. This treatment seems to be effective also in case of endovascular treatment. AAA treatment in SVI should be performed (especially in elective settings) in high volume centers where it is possible to bring on collaboration across different surgical specialists.

Keywords situs viscerum inversus, aortic aneurysm, surgery

1. Introduction

Situs viscerum inversus (SVI) is a rare congenital condition in which the organ's position is completely reversed, affecting both abdominal and thoracic compartments (*1,2*). The incidence of SVI ranges between 1:5,000 and 1:20,000 of newborns (*1*), with no evidence of gender inheritancy (*3*). This condition is due to a complete failure of the normal left-right asymmetry (*2*), even if the exact SVI etiology is yet unclear (*3*). To date, the most widely accepted theory is the deregulation of Sonic Hedgehog (Shh) protein during the third week of embryonic development (*4*), while it is known that the genetic deletions in KIF3-A or KIF3-B are responsible of alterations in visceral disposition within the abdomen/thorax (*2*).

Six risk factors were recently identified for SVI (*2*): *i*) family history of heart defects, *ii*) family history of noncardiac anomalies, *iii*) maternal diabetes, *iv*) antitussive use during pregnancy, *v*) paternal smoking, and *vi*) low socioeconomic status. Furthermore, SVI could be an incidental condition or could occur in conjunction with several pathological conditions such as primary ciliary dyskinesia, polysplenia, Ivermark's syndrome. The association between SVI and vascular anomalies are rareand, in particular, the prevalence of abdominal aortic aneurysm (AAA) in SVI patients is currently unknown. When SVI is not associated with the anamnestic history of the patient, the preoperative study and the surgical procedure are not standardizable.

The aim of this systematic review is to highlight how AAA is diagnosed and treated when associated with SVI.

2. Literature search strategy

We conducted a systematic review of published studies in accordance with Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) guidelines (*5*) to identify studies reporting abdominal aortic aneurysm treatment in patients with SVI.

The population, intervention, comparator, outcome, and study design (PICOS) criteria are reported in Supplemental Table S1 (*https://www.irdrjournal.com/supplementaldata/178*). Supplemental Table S2 (*https://www.irdrjournal.com/supplementaldata/178*) reports the National Institutes of Health (NIH) quality assessment for each study considered for this research.

PubMed/MEDLINE, EMBASE, Web of Science (WOS), Google Scholar databases were surveyed using a combination of the following search terms: "situs viscerum inversus AND abdominal aortic aneurysm", "situs inversus AND aortic abdominal aneurysm", "situs organum inversus AND abdominal aortic aneurysm". The survey includes all publications until 30 June 2023.

All publications considering the AAA treatment in patients with SVI in both elective or emergency settings were considered for further selection. Full-text publications considered for inclusion were reviewed by two independent assessors (Paolo Ossola and Diego Coletta), and the references therein were further scrutined to find additional publications unreported in the previous survey. Only publications in English, Spanish or Italian language were selected. All concerns in the publications selected were discussed and consensus was sought across all authors. When agreement was not found, the publication of concern was excluded from the selection pool.

In accordance with the pre-established agreement, extracted data included: study design, country where the study was performed, date of publication, number of patients, age, gender, type of surgical procedure, intraoperative and postoperative complications, length of hospital stay (LOS), and follow-up. Results are reported as descriptive statistics with mean and minimum-maximum range.

3. Studies selection

The first literature survey resulted in 326 papers. After the first screening, 295 publications were excluded because off-topic title and abstract or duplicate. Eventually, 12 full-text publications (*4,6-16*), accounting for 12 patients, met the selection criteria and are included in our review (Figure 1): 10 are written in English (*4,7,8,10-16*), one in Spanish (*6*), and one in Italian (*9*).

4. Characteristics of studies

The studies selected are conducted in various countries: 5 studies are in Europe (*4,7-10*), 1 in the United State of America (*11*), 2 in Canada (*12,13*), 1 in China (*14*), 2 in Japan (*15,16*), and one in Colombia (*6*).

All the studies are case reports of a single patient. Surgery procedures are performed in general surgery division in 2 reports (*4,10*), vascular surgery departments in 5 cases (*7-9,11,14*), and 3 cases are treated in cardio-vascular departments (*13,15,16*). In 2 cases (*6,12*), a team of vascular and general surgery specialists performed the procedure.

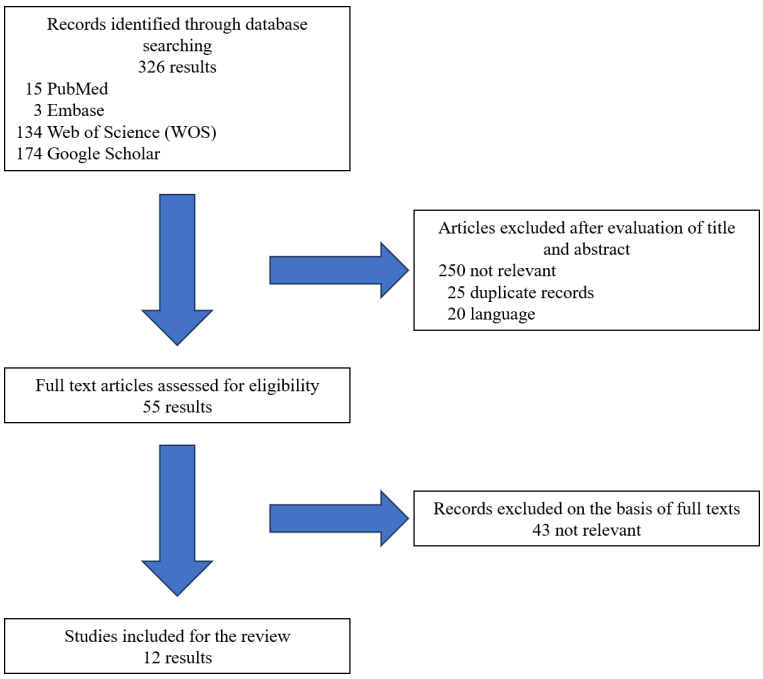

Figure 1. PRISMA flow-chart for studies selection.

5. Demographic findings

Demographic findings are reported in Table 1.

Pathological anamnesis is reported in 7 studies (*4,6-8,12,14,16*): 7 patients are affected by arterial hypertension (*4,6-8,12,14,16*), and two cases also by ischemic heart disease previously (*14,16*). AAA size is reported in 10 studies (*4,6-9,11,12-15*): the mean AAA diameter was 70.5 mm (range: 55-90 mm); also AAA localization is reported in all studies, and summarized in Figure 2. The most common localization in normal conformed patients is infrarenal aortic portion, and juxtarenal aneurysm accounts for about 15% (*17*). Our series, accounting 8 infrarenal aneurysms (*4,6,8,10,11,13-15*), 1 juxtarenal (*12*), 1 pararenal (*7*) and two infrarenal aneurysms associated with left common iliac artery aneurysm (*9,16*), didn't differ from data derived from normal conformed patients.

SVI was a known condition in 8 cases (*4,6,7,11-15*); in 3 patients (*8,9,16*), SVI were identify by a computed tomography scan contrast enhanced (CT) performed for prostate hypertrophy study (*8*), and for abdominal pain study (*9,16*). In one case (*10*), SVI was diagnosed by abdomen ultrasound scan (US) in a patient with abdominal pain.

6. Operative findings

The present review analyzed studies involving surgery in both elective and emergency settings: 6 studies reported data on elective surgery (*4,7,8,11,13,14*), and 6 on emergency procedures (*6,9,10,12,15,16*).

No intraoperative complications are reported. In 3 cases, patients experienced postoperative complications: a case of acute distal femoral, popliteal and pedidia right arteries ischemia during the immediate postoperative period treated with thromboendarterectomy (*6*); a respiratory failure associated with deep vein thrombosis of the right leg treated with warfarin therapy (*15*), and postoperative fever (*13*). All postoperative complications are reported in patients undergoing emergency surgery.

LOS is reported in 6 studies (*4,7,9,12,14,15*) with mean LOS of 12.5 days and a range of 3–46 days. Data on postoperative follow-up are reported in 8 studies (*4,6-8,13-16*) with a medium observation period of 13.5 months (range: 3–60)

7. Discussion

This systematic review focuses on the actual AAA treatment in patients with SVI. The extreme unlikelyhood

Table 1. Demographic findings of Literature

Author (*Ref*), Year	Country	Sex	Age (Years)	SIT known	Diagnosis of AAA	AAA dimension (mm)	AAA localization
Bonnelly (*4*), 2022	Spain	M	58	Yes	CT	56	infrarenal
Téllez-Beltrán (*6*), 2020	Colombia	F	56	Yes	US + CT	80	infrarenal
Gatta (*7*), 2020	Italy	M	73	Yes	US + CT	60	pararenal
Cwinn (*12*), 2016	Canada	F	82	Yes	CT	90	iuxtarenal
Riera Hernándeza (*8*), 2015	Spain	M	69	No	CT	60	infrarenal
Chan (*14*), 2010	China	M	81	Yes	CT	75	infrarenal
Kimura (*15*), 2008	Japan	F	80	Yes	CT	85	infrarenal
Kato (*16*), 2006	Japan	M	75	No	CT	na	infrarenal + left common iliac artery
Baccellieri (*9*), 2006	Italy	M	70	No	CT	55	infrarenal + left common iliac artery
Occhionorelli (*10*), 1998	Italy	F	76	No	US+CT	na	infrarenal
Huston (*11*), 1990	USA	M	84	Yes	CT	70	infrarenal
Ricci (*13*), 1989	Canada	M	79	Yes	US + arteriography	74	infrarenal

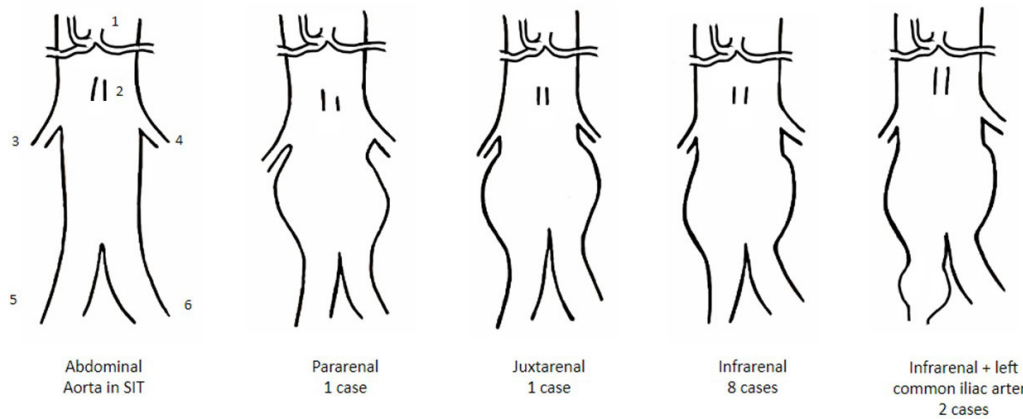

Figure 2. Conformation of normal aorta in SVI and type of AAA in SVI reported in Literature. 1: celiac trunk; 2: superior mesenteric artery; 3: left renal artery; 4: right renal artery; 5: left common iliac artery; 6: right common iliac artery.

of this condition is evident in the low literature studies on the topic: in our survey we identified only 12 studies (*4,6-16*) reporting data on 12 patients.

7.1. Clinical presentation

In people with normal visceral anatomy, the clinic presentation of AAA varies: in some cases it could be completely asymptomatic, in this situation AAA is an incidental finding during routine exams. In fact, AAA is due to a progressive enlargement of the abdominal aortic diameter, with the stretch of all three artery layers, determining a thin and weak aortic wall (*18*). In other cases, patients complain of abdominal pain or hemorrhagic shocks: the main complication of AAA, and in general of an artery aneurysm, is its rupture with hemoperitoneum (or hemo-retroperitoneum) with hemorrhagic shock and death (*19*).

SVI could represent only a variation of a normal anatomy with no alteration in life and health expectancy of the patient being an occasional finding during routine clinical exams. Otherwise, SVI could be an aspect of a group of pathological conditions defined as "syndromic conditions". The most common syndromic conditions are primary ciliary dyskinesia, polysplenia, and Ivermark's syndrome.

Cardiac malformations are not infrequent in SVI patients (3–9%) (*2*); while the most common vascular anomalies are the interrupted inferior vena cava (20%) and preduodenal portal vein (42%) (*2*). In the rare case of SVI, AAA could appear also as an intestinal occlusion (*20*).

7.2. Diagnosis

AAA diagnosis is obtained through US and contrast enhancement CT: obviously, the same techniques allow to detect any anomalies in the organs topology in the body. So, often, AAA and SVI are diagnosed at the same time.

US is a first level tool able to identify AAA, its localization along the aorta, describe the diameter, the status (*i.e.* the rupture or the fissuring), and the blood flow inside the aneurysm sac. US is fast, does not need injection of any contrast fluids, and does not expose the patient to ionizing radiations. The main limit of US is that the sensibility of this technique is linked to the operator ability; nevertheless, US is a gold standard in the follow-up, particularly in case of young or female patients, avoiding radiation exposure (*21*). CT scan with vascular acquisitions is mandatory in the definitive description and for treatment planning. If AAA is associated with SVI, CT allows to identify the anomalies in organs location, to verify the presence of vascular anomalies, that is, anomalies in vein or artery number, or course.

In the literature, 11 cases (*4,6-12,14-16*) underwent preoperative CT, in one case AAA was diagnosed with US and arteriography (*13*): this last case was treated in1989 when CT was not largely available. CT remains the most relevant imaging method for AAA and SVI diagnosis at the same time.

7.3. Treatment

In patients with normal disposition of internal organs, unruptured AAA is treated when the diameter is ≥ 5.5 cm in male or ≥ 5 cm in female or when the growth rate is ≥ 0.5 cm in 6 months (*22*). When ruptured, AAA treatment is mandatory in emergency settings. In case of SVI, the indications for AAA treatment are the same as in normal patients.

In the surveyed publications, the mean AAA diameter was 70.5 mm (range: 55–90 mm), 6 cases (*6,9,10,12,15,16*) were treated in emergency settings, and 6 (*4,7,8,11,13,14*) cases were planned as elective procedures. Technical approaches for AAA vary: in this work 11 cases (*4,6-13,15,16*) were treated with open approach with aneurysmectomy and graft interposition. Chan *et al.* (*14*) reported the first case of AAA in SVI endovascular repair: the 81 years-old patient with a medical history of SVI and previous abdominal surgery for colon cancer, was treated with a Cook Zenith endovascular device and discharged on 3rd postoperative day without complications. Chan *et al.* (*14*) affirmed that endovascular repair is safe and feasible also in case of SVI, with not specific difficulties compared to normal-topology patients. Certainly, endovascular approaches should be reserved in high expertise and large volume vascular Departments.

Laparotomic approach for AAA in SVI patients was performed by general and vascular surgeons, especially in emergency conditions. In our series 2 cases (*6,12*) were treated by a multidisciplinary surgical team consisting of general and vascular surgeons, 5 patients (*7-9,11,14*) by vascular surgeons, 2 cases (*4,10*) by general surgeons, 3 cases (*13,15,16*) by cardio-vascular surgeons. The possibility to treat complex cases by multispecialistic teams (*e.g.* general and vascular surgery, cardio-vascular surgery, or other), represent an advantage in terms of safety and good results of the surgical procedure.

7.4. Complications

Interestingly, no intraoperative complications are registered: this finding could be explained considering that AAA in SVI is a very rare condition and preferably treated in high volume and expertise centers. On the other hand, because AAAs in SVI are treated also in emergency settings, the absence of intraoperative complications could reflect the absence of technical differences compared to AAA in normal conformed patients.

Postoperative complications are registered in emergency conditions: Beltran *et al.* (*6*) reported a case of acute distal femoral-popliteal and pedidia right arteries ischemia treated with thromboembolectomy throw the femoral artery with complete ischemia resolution. The patient described by Kimura *et al.* (*15*) developed respiratory failure and deep vein thrombosis of the right leg; the patient was extubated on 14 postoperative day with anticoagulation therapy with warfarin continued for the deep vein thrombosis. Ricci *et al.* (*13*) reported a postoperative course complicated by transitory fever, that could be due to the body reaction to the graft.

Operative time is reported in only 2 studies: Beltran *et al.* (*6*): 96 minutes and Kimura *et al.* (*15*) 190 minutes: for this reason it is not possible to know if SVI increases the operative time. The median LOS was 12.5 days (range 3–46) and is reported in 6 studies (*4,7,9,12,14,15*).

7.5. Follow-up

Follow-up is mandatory to early identify graft leak, the graft thrombosis, or rupture; in our review the mean follow-up resulted in 13.5 months (range: 3–60), and was reported in 8 studies (*4,6,7,8,13-16*). CT is the gold standard for imaging follow-up, even if also US could be adopted in order to reduce radiological exposition. As of today, no specific protocols are available for AAA in SVI follow-up.

8. Conclusion

The analysis of the scientific literature shows that the treatment of AAA in SVI is feasible and does not show an incremented morbidity compared to patients with a normal visceral configuration. This treatment seems to be effective also in case of endovascular treatment. The preoperative imaging study with CT is mandatory before performing any invasive treatment in SVI patients, in order to identify potential alteration in the vessels course. However, the AAA treatment in SVI should be performed (especially in elective settings) in high volume centers where it is possible to bring on collaboration across different surgical specialists, such as general, vascular and cardio-vascular surgeons. Further studies are necessary to explore the role of endovascular techniques in SVI.

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**Address correspondence to:*
Paolo Ossola, Department of Surgery, Cardinal Massaia Hospital, Corso Dante Alighieri, 202, 14100 Asti, Italy.
E-mail: paolo.ossola90@gmail.com

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Systematic analysis and evaluation of chromosome aberrations in major birth defects associated with infertility

Fuying Lan[1], Zhongzhong Chen[1,2,*], Xiaoling Lin[1,*]

[1] Department of Urology, Shanghai Children's Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China;
[2] Urogenital Development Research Center, Shanghai Children's Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China.

SUMMARY Previous studies have indicated an elevated risk of infertility in certain birth defects, including congenital heart disease (CHD), hypospadias, cryptorchidism, and disorders of sexual development (DSD). Although the identification of chromosomal abnormalities or chromosomal aberrations (CAs) is crucial for the diagnosis of these conditions, the assessment of CAs in these disorders remains unclear, and few large-scale studies have been conducted at multiple centers. The aim of the current study was to systematically evaluate the prevalence of CAs in CHD, hypospadias, cryptorchidism, and DSD. Studies reporting CAs in these birth defects were retrospectively analyzed from 1991-2023, using online databases such as PubMed and Google scholar as well as preprints and references from related literature. Comprehensive screening, data acquisition, and systematic assessments of the identified literature were performed. Ultimately, searches yielded a total of 7,356 samples from 14 published articles on CHD, 298 hypospadias cases from 4 published articles, 1,681 cryptorchidism cases from 4 published articles, and 2,876 DSD cases from 7 published articles. Carrier rates of CAs varied widely among these studies and conditions. A retrospective analysis revealed that CHD was associated with the highest carrier rate (26%) for CAs, followed by DSD (21%), hypospadias (9%), and cryptorchidism (5%). A subtype analysis of CAs indicated a higher prevalence of numerical abnormalities among the reported cases. Therefore, considering CAs in birth defects associated with infertility is imperative. This provides a foundation for the further clinical implementation of chromosomal screening and enhancing high-risk screening for individuals in the real world.

Keywords chromosomal aberration, birth defects, infertility

1. Introduction

Birth defects are a physiological or structural abnormality at birth that can arise from various factors, including developmental defects, infection, and heredity. Approximately 6% of newborns worldwide are affected by birth defects, ranging from treatable cases to severe conditions that significantly impact lifelong health (*1*). This issue has become a crucial determinant of population quality, particularly as several countries have experienced a significant decline in birth rates over the past few decades. In China, for example, the birth rate has noticeably decreased from 14‰ to 6.8‰ since 2000, accompanied by a decline in fertility and a changing profiling of major birth defects (*2*). Birth defects such as congenital heart disease (CHD) and hypospadias are associated with infertility (*2*), uncovering factors contributing to reduced fertility rates. In addition, disorders that affect the endocrine and reproductive systems such as androgen insensitivity syndrome (AIS) (*3*), a common disorder of disorders of sex development (DSD), may also have significant impacts on fertility. Chromosomal aberrations (CAs), consisting of numerical and structural chromosome abnormalities, are a common characteristic of severely clinical consequences such as spontaneous abortion, death, and birth defects (*4,5*). CAs are also associated with disorders of reproductive and sexual development (*5,6*). Numerical CAs, mainly including aneuploidy, are caused by chromosome segregation errors in mitosis, whereas structural CAs in the form of chromosomal fragments, rearrangements, chromosomal translocations, and heterozygous deletions are the result of DNA damage and arm-level chromosome gain or loss (*7,8*). Despite many studies focusing on the identification of associated genes using whole-exome sequencing, there remains a lack of multicenter studies on CAs. Therefore, the aim of the current study was to systematically assess the prevalence of CAs in these four birth defects that affect fertility, offering fundamental insights for the future clinical

implementation of chromosomal screening.

2. Literature search strategies and analytical methods

2.1. Search strategies

This systematic review was conducted following the PRISMA guideline (*9*). PubMed and Google scholar were used to search for articles published between 1991 and 2023 by two authors with the keywords "chromosome aberrations", "chromosomal disorder", "congenital heart disease", "hypospadias", "cryptorchidism", and "disorders of sex development". In addition, the references of the articles were reviewed to identify potentially relevant studies and ensure comprehensive coverage of the relevant literature. Only English-language literature was considered for analysis. Two reviewers independently evaluated the eligibility of the articles based on the following criteria: (a) assessment of the association between these types of birth defects and CAs; (b) availability of original data from randomized controlled trials, non-randomized controlled clinical trials, prospective and retrospective cohort studies, and case-control studies; and (c) the incidence of CAs for which data were available or could be obtained from the given data. Potentially eligible studies were excluded if they were: (a) conference papers or editorials; (b) case reports and case series; (c) repetitions and literature with missing or insufficient information, and (d) articles published before 1990. A full-text review was subsequently conducted for data extraction from all remaining studies. The name of the first author, year of publication, source, sample size, and number of CAs were recoded for each included study. In cases where a consensus could not be reached on study eligibility, a third reviewer was consulted.

2.2. Data analysis

Both a common-effect model and random-effects model were used to provide pooled estimates of the incidence of these four birth defects. The common-effect model assumes uniformity of the true effect across studies, while the random-effects model accounts for substantial variation in effect sizes by incorporating it into the standard errors. While we are confident in the comprehensiveness of our search strategy, not all conducted studies may have been included. The random-effects model addresses this by assuming that our sample is representative of all existing studies, including those that may not have been published or completed. For each complication of interest, the pooled incidence was computed along with a 95% confidence interval (CI). The I^2 statistic was used as an indicator of study heterogeneity. The I^2 statistic was reported for each pooled estimate as a measure of study heterogeneity, with values of $p \geq 0.100$ and $I^2 \leq 25.0\%$ indicating good homogeneity between studies and an I^2 greater than 50% suggesting substantial study heterogeneity. Event rates were calculated in R with the metafor package (*10*).

3. Results

3.1. Summary of the systematic literature search

Figures 1-4 depict the PRISMA diagrams illustrating the systematic literature retrieval process for CHD, hypospadias, cryptorchidism, and DSD, respectively. Table 1 provides a summary of the outcomes documented in the included publications concerning these conditions. All studies included in the analysis were published between 1991 and 2023.

For CHD, a total of 622 records were included in the initial search after removing duplicate articles (Figure 1). Ultimately, 14 publications met the inclusion criteria, and 7,356 cases were included. The incidence of CAs ranged from 9-59%.

For hypospadias, the initial search resulted in 297 records after removing duplicate articles (Figure 2). Ultimately, 4 publications met the inclusion criteria, and 298 cases were included. The incidence of CAs ranged from 4-12%.

For cryptorchidism, a total of 275 records were included in the initial search after removing duplicate articles (Figure 3). Ultimately, 4 publications met the inclusion criteria, and 1,681 cases were included. The incidence of CAs ranged from 3-8%.

For DSD, a total of 67 records were included in the initial search after removing duplicate articles (Figure 4). Eventually, 7 publications met the inclusion criteria, and 2,876 cases were included. The incidence of CAs ranged widely from 10-43%.

3.2. Incidence of CAs

The combined results of the incidence of CAs in CHD, hypospadias, cryptorchidism, and DSD was also calculated (Table 1). Fourteen studies reported CAs in CHD, resulting in a total of 1,204 CA events out of 7,356 included cases. The total pooled incidence of CAs for CHD was 26% (95% CI, 19-34%, $I^2 = 97\%$; Figure 5).

For hypospadias, a total of 26 CAs were detected in 298 included cases. The overall pooled incidence of CAs for hypospadias was 9% (95% CI, 6-13%, $I^2 = 19\%$; Figure 6).

Four studies reported CAs in the context of cryptorchidism, with a total of 78 CA events in 1,681 included cases. The total pooled incidence of CAs for cryptorchidism was 5% (95% CI, 3-7%, $I^2 = 83\%$; Figure 7).

For DSD, four studies reported CAs, resulting in 567 CA events out of 2,876 included cases. The total pooled incidence of CAs for DSD was 21% (95% CI, 14-31%, $I^2 = 97\%$; Figure 8).

Table 1. Summary of study characteristics and CA carrier results in the included studies

Diseases	PMID	Authors (Year published)	Incidence (%)	N	Sample size
CHD	12797095	Allan *et al*. (1991)	16	77	467
	36011280	Okashah *et al.* (2022)	59	16	27
	35169781	Atli *et al.* (2021)	22	5	23
	34988127	Zhang *et al*. (2021)	26	279	1,089
	34098741	Chelliah *et al*. (2021)	46	37	80
	18512234	Rosa *et al*. (2008)	14	29	204
	33345990	Mustafa *et al*. (2020)	37	80	217
	33247990	Tomotaki *et al*. (2021)	56	25	45
	32371943	Qiu *et al*. (2020)	20	48	235
	30558042	Cai *et al*. (2018)	13	19	146
	30133550	Luo *et al*. (2018)	39	140	362
	25497206	Stoll *et al*. (2015)	9	354	4,005
	24145389	Trevisan *et al*. (2013)	17	50	298
	1590238	Smythe *et al*. (1992)	28	45	158
	Current study		26[a]	1,204	7,356
Hypospadias	12394752	Moreno-Garcia *et al*. (2002)	7	7	100
	29473028	González *et al*. (2018)	4	2	49
	1514208	Yabumoto *et al*. (1992)	12	16	131
	1686509	Yamaguchi *et al.* (1991)	6	1	18
	Current study		9[b]	26	298
Cryptorchidism	12394752	Moreno-Garcia *et al*. (2002)	3	26	916
	1686509	Yamaguchi *et al.* (1991)	5	4	83
	8738627	Sasagawa I *et al*. (1996)	4	7	160
	32293821	Sharifi N *et al*. (2020)	8	41	522
	Current study		5[a]	78	1,681
DSD	34036105	Benchikh *et al*. (2021)	15	154	1,005
	29581155	Kohva *et al*. (2018)	37	204	550
	22644991	Öcal *et al*. (2012)	27	78	285
	32282607	Dhamankar *et al*. (2020)	23	7	30
	29996319	Yi P *et al*. (2018)	43	23	53
	36419940	Man E *et al*. (2023)	11	68	607
	27754965	Ganie *et al*. (2016)	10	33	346
	Current study		21[a]	567	2,876

PMID, PubMed Unique Identifier; CAs, chromosomal abnormalities or chromosomal aberrations; N, number; CHD, congenital heart disease; DSD, disorders of sexual development; [a], incidence was calculated based on a random effects model; [b], incidence was calculated based on a common effect model.

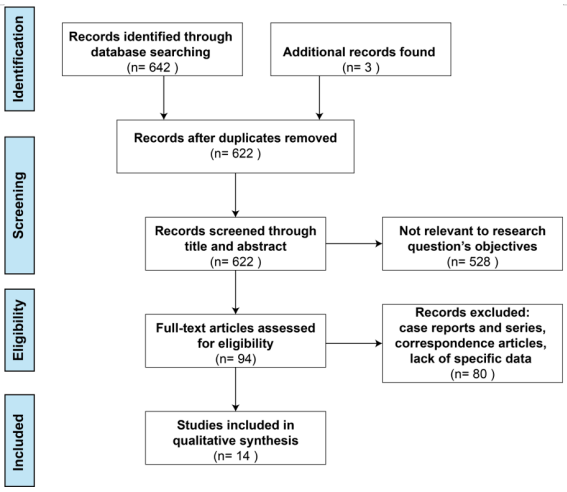

Figure 1. PRISMA flowchart of a systematic literature search for CHD.

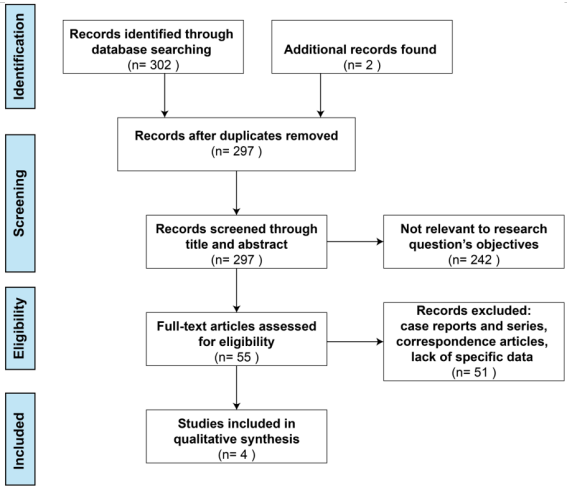

Figure 2. PRISMA flowchart of a systematic literature search for hypospadias.

3.3. Subtypes of CAs in fertility-related birth defects

Table 2 shows the subtypes of CAs detected in four birth defects affecting fertility, excluding cases without specific subtypes of CAs. As shown in the table, there was a higher frequency of autosomal chromosome

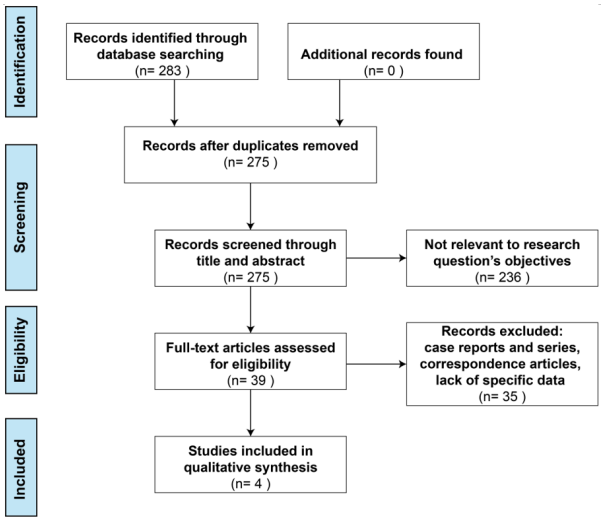

Figure 3. PRISMA flowchart of a systematic literature search for cryptorchidism.

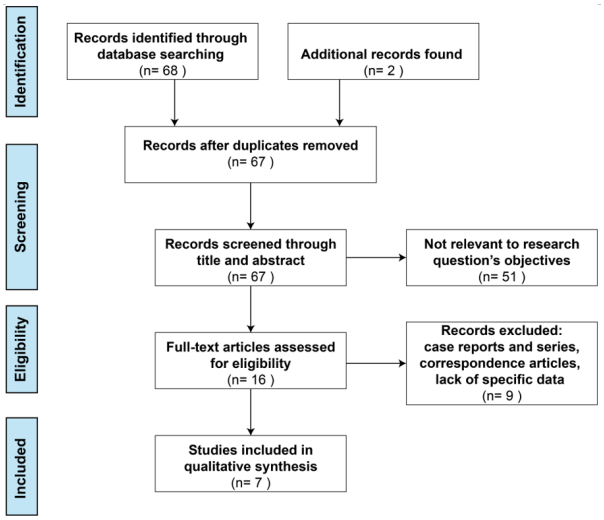

Figure 4. PRISMA flowchart of the systematic literature search for DSD.

abnormalities in CHD cases compared to sex CAs (94.80% *vs.* 5.20%, respectively). Among autosomal CAs in CHD cases, numerical abnormalities constituted the majority (70.65%), with trisomy 21 and trisomy 18 being the most common subtypes of CAs, accounting for 40.96% and 22.70%, respectively. In contrast to CHD, there was a higher incidence of sex CAs in patients with DSD, with 92.59% of cases exhibiting numerical abnormalities. Notable subtypes of CAs in DSD included Turner syndrome, Klinefelter syndrome, and mixed gonadal dysgenesis, respectively accounting for 37.04%, 26.10%, and 29.45%.

The studies on hypospadias and cryptorchidism yielded limited information on karyotype patterns, with sex CAs accounting for 69.23% and 60.26%, respectively; numerical abnormalities were the major subtype. In the context of sex chromosome numerical abnormalities in hypospadias and cryptorchidism, Klinefelter syndrome was the predominant type,

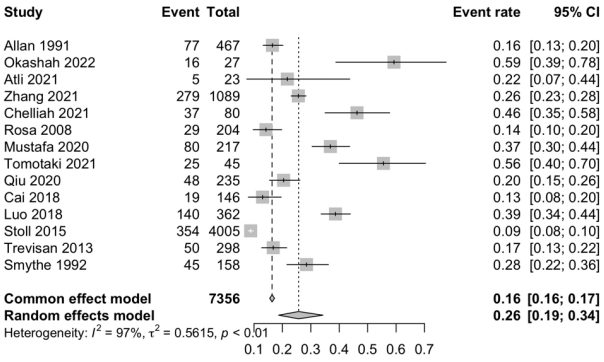

Figure 5. Forest plot and pooled analysis of CA events in CHD

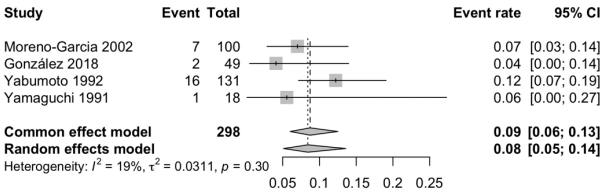

Figure 6. Forest plot and pooled analysis of CA events in hypospadias.

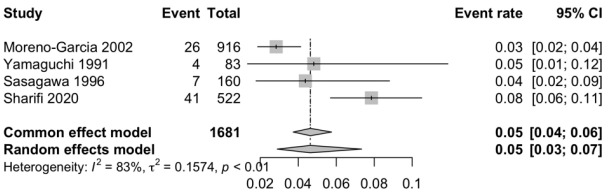

Figure 7. Forest plot and pooled analysis of CA events in cryptorchidism.

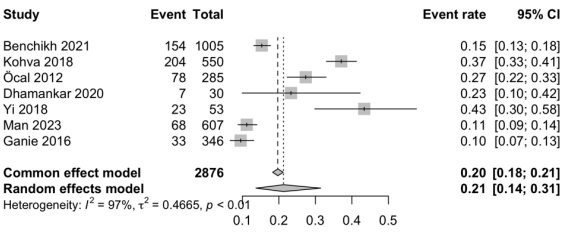

Figure 8. Forest plot and pooled analysis of CA events in DSD.

respectively accounting for 19.23% and 51.28%.

4. Discussion

Over the past two decades, there have been significant advancements in prenatal diagnosis technology, coinciding with notable shifts in the profiling of birth defects. CHD and hypospadias, both of which increase the risk of infertility, have progressively emerged as the major birth defects (*2*). CAs represent a common genetic cause of birth defects, exhibiting considerable diversity in samples across different regions for CHD, hypospadias, cryptorchidism, and DSD (Table 1). However, there has been a lack of a systematic assessment of the impact of CAs on these four birth defects, and a dearth of

Table 2. The distribution of types of chromosome abnormalities in four birth defects

Classification	CHD		Hypospadias		Cryptorchidism		DSD	
	N	%	N	%	N	%	N	%
Autosomal chromosome abnormalities	1,111	94.80	8	30.77	31	39.74	42	7.41
Numerical abnormalities	828	70.65	1	3.85	6	7.69	5	0.88
Trisomy 18	266	22.70	1	3.85	1	1.28	0	0.00
Trisomy 21	480	40.96	0	0.00	1	1.28	1	0.18
Trisomy 13	75	6.40	0	0.00	0	0.00	0	0.00
Others	7	0.60	0	0.00	4	5.13	4	0.71
Structural abnormalities	170	14.51	1	3.85	25	32.05	37	6.53
Translocations	21	1.79	1	3.85	8	10.26	16	2.82
Duplications	6	0.51	0	0.00	1	1.28	0	0.00
Deletions	103	8.79	0	0.00	1	1.28	3	0.53
Inversions	3	0.26	0	0.00	13	16.67	18	3.17
Ring-chromosome	3	0.26	0	0.00	0	0.00	0	0.00
Chromosomal polymorphisms	9	0.77	0	0.00	2	2.56	0	0.00
Others	25	2.13	0	0.00	0	0.00	0	0.00
Not classified*	113	9.64	6	23.08	0	0.00	0	0.00
Sex chromosome abnormalities	61	5.20	18	69.23	47	60.26	525	92.59
Numerical abnormalities	34	2.90	7	26.92	46	58.97	525	92.59
Klinefelter syndrome	2	0.17	5	19.23	40	51.28	148	26.10
Turner syndrome	30	2.56	0	0.00	6	7.69	210	37.04
Triple X syndrome	2	0.17	0	0.00	0	0.00	0	0.00
Mixed gonadal dysgenesis	0	0.00	2	7.69	0	0.00	167	29.45
Structural abnormalities	1	0.09	0	0.00	1	1.28	0	0.00
Deletions	0	0.00	0	0.00	1	1.28	0	0.00
Inversions	1	0.09	0	0.00	0	0.00	0	0.00
Not classified*	26	2.22	11	42.31	0	0.00	0	0.00
Total (excluded ambiguous)	1,172	100	26	100.00	78	100.00	567	100.00

N, number; CAs, chromosomal abnormalities or chromosomal aberrations; CHD, congenital heart disease; DSD, disorders of sexual development.
*The studies provided only the autosomal or sex CA information in cases but no further detailed information about the subtype of CAs.

multicenter studies examining CAs persists. As a result, our focus was directly on these four birth defects, all of which could potentially affect fertility in adulthood. A systematic evaluation of the prevalence of CAs and their subtypes in these disorders was performed.

Results revealed a high incidence of CAs in genomes associated with fertility-related diseases. The respective prevalence of CAs in CHD, hypospadias, cryptorchidism, and DSD was 26%, 9%, 5% and 21%, with CHD exhibiting the highest prevalence and cryptorchidism the lowest. CAs can result in severe phenotypes, such as CHD and DSD. CAs are categorized into numerical and structural abnormalities based on the mechanisms of chromosome segregation errors or DNA damage (*7,8*). Numerical CAs were the predominant anomalies observed in cases of CHD, cryptorchidism, and DSD, respectively accounting for 73.55%, 66.67%, and 93.47%. Numerical CAs typically involve deviations in the number of chromosomes from the normal karyotype due to misallocation during mitosis or cell division blockage. Numerical CAs mainly include aneuploidy, triploidy, and tetraploidy in humans. Given that systemic triploidy is usually fatal for humans, triploid fetuses typically result in eventual abortion (*11*). Aneuploidy, characterized by the gain or loss of chromatid or chromosome regions, represents the primary form of numerical CAs in clinical settings, contributing to conditions such as trisomy 21, trisomy 18, and Turner syndrome, which were found to be prevalent in CHD and DSD cases. In cases of CAs related to CHD, numerical abnormalities were frequently observed in euchromosome, accounting for 70.65%. And in DSD cases of CAs, the vast majority (92.59%) involved the sex chromosomes.

Numerical CAs of DSD are frequently associated with conditions such as Turner syndrome, Klinefelter syndrome, Triple X syndrome, Jacob's syndrome, mixed gonadal dysgenesis, and chimerism. Turner syndrome represents the most prevalent sex-related CAs and is the major genetic cause of primary amenorrhoea in women. Its typical karyotype is 45,X, but variations can include karyotypes such as 45,X/46,XX, 45,X/46XY, and 45,X/47,XXX (*12*). Clinical presentations often include a short stature, gonadal insufficiency, primary or secondary amenorrhea, infertility, micrognathia, low-protruding ears, a short neck, and elbow ectropion (*13,14*). Klinefelter's syndrome usually manifests with a chromosomal karyotype of 47,XXY, and variants can include karyotypes such as 48,XXXY, 49,XXXXY, and 46,XY/47,XXY (*15*). Patients may exhibit a tall stature, narrow shoulders, wide hips, sparse body hair, gynecomastia, small testes, androgen deficiency, learning disabilities, and delayed speech development (*13*). In Triple X syndrome with a karyotype 47,XXX, patients may clinically exhibit increased height and an elevated risk of learning disabilities, delayed speech, language, and motor skills development, weak muscle tone, behavioral and emotional difficulties, seizures,

and renal abnormalities (*14,15*). However, some cases of Jacob's syndrome may not present with any discernible phenotypic abnormalities. Jacob's syndrome is characterized by a karyotype of 47,XYY. Clinically, patients with this syndrome typically have an increased height, an elevated risk of learning disabilities, delayed development of speech, language, and motor skills, dystonic weakness, hand tremors, seizures, asthma, scoliosis, as well as behavioral and emotional difficulties (*14,15*). Similar to triple X syndrome, certain cases of Jacob's syndrome may present without noticeable phenotypic anomalies.

45,X/46,XY is a mixed gonadal hypoplasia syndrome characterized by a wide range of clinical phenotypes, spanning from females with Turner syndrome to phenotypically normal males with genital abnormalities and a short stature. Gonadal function appears to be adequate for spontaneous puberty in most 45,X/46,XY males. The etiology of its development is yet to be fully understood, although a widely accepted hypothesis suggests that it results from the nondisjunction or structural rearrangement of the Y chromosome during either fertilized egg division or early embryonic cell division (*16*). During mitosis, a delayed separation or rearrangement of the Y chromosome can generate three distinct cell lineages, 45,X, 46,XY, and 47,XYY, with trisomic cells often being lost in subsequent divisions, while 45,X and 46,XY cells are more likely to persist. During the segregation of sister chromatids of the Y chromosome, a break may occur in the palindrome or inverted repeat region, followed by a homologous exchange of sister chromatids, leading to structural abnormalities of the Y chromosome if this process is disrupted.

Structural CAs encompass a variety of alterations, such as deletions, duplications, inversions, translocations, insertions, isochromosomes, ring chromosomes, and chromosomal polymorphisms. Although not the primary form of CAs in fertility-related birth defects, chromosomal structural abnormalities account for 33.33% of CAs in cases of cryptorchidism. Chromosomal polymorphisms are minor structural aberrations in human chromosomes, primarily occurring in regions with highly repetitive DNA sequences but not in coding regions. Generally considered to have no clinical effects, these polymorphisms tend to emerge in highly repetitive sequences with no transcriptional activity. However, a recent study has suggested that chromosomal polymorphisms within the heterochromatin region could potentially lead to abnormalities during chromosome division and gamete production, ultimately resulting in abnormal embryonic development (*17*). Nevertheless, such chromosomal polymorphisms are rarely associated with fertility-related birth defects, accounting for only 0.77% of CAs in individuals with CHD and 2.56% of CAs in individuals with cryptorchidism. Such chromosomal polymorphisms were not found in the other two disorders. The most recent data on the impact of chromosomal polymorphisms on the reproductive outcomes of couples undergoing intracytoplasmic sperm injection (ICSI) treatment, based on 929 fresh and frozen embryo transfer cycles involving 692 women, did not reveal any disparities in reproductive outcomes between carriers and non-carriers of any type or number of chromosomal polymorphisms (*18*).

This retrospective analysis may be limited by the limited data available in the included literature. First, the analysis is influenced by the inclusion of studies with small sample sizes rather than large cohort populations. Second, the exclusion of certain articles due to unavailable or incomplete data could result in the omission of important studies.

The current study aimed to investigate the connections between fertility-related birth defects and CAs. Despite the reduced emphasis on chromosomal analysis, guidelines still recommend its utilization. The results of this study highlight its effectiveness in studying reproductive disorders. In addition, chromosome analysis in individuals diagnosed with reproductive diseases plays a significant role in the expanding field of preimplantation genetic diagnosis (PGD).

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**Address correspondence to:*
Zhongzhong Chen and Xiaoling Lin, Department of Urology, Shanghai Children's Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200062, China.
E-mail: zhongzhongchen@gmail.com; lxling.85@163.com

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Original Article

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Haploinsufficiency of *NKX2-1* is likely to contribute to developmental delay involving 14q13 microdeletions

Osamu Machida[1,2], Haruko Sakamoto[3,4], Keiko Shimojima Yamamoto[5,6], Yuiko Hasegawa[7], Satoi Nii[4], Hidenori Okada[3], Kazuki Nishikawa[3], Shin-Ichi Sumimoto[4], Eriko Nishi[7], Nobuhiko Okamoto[7], Toshiyuki Yamamoto[1,5,*]

[1] Division of Gene Medicine, Graduate School of Medicine, Tokyo Women's Medical University, Tokyo, Japan;
[2] Department of Pediatrics, Tokyo Women's Medical University, Tokyo, Japan;
[3] Department of Pediatrics, Japanese Red Cross Osaka Hospital, Osaka, Japan;
[4] Otemae Rehabilitation Center for Children, Japanese Red Cross Osaka Hospital, Osaka, Japan;
[5] Department of Transfusion Medicine and Cell Processing, Tokyo Women's Medical University, Tokyo, Japan;
[6] Institute of Medical Genetics, Tokyo Women's Medical University, Tokyo, Japan;
[7] Department of Medical Genetics, Osaka Women's and Children's Hospital, Osaka, Japan.

SUMMARY Nucleotide variations or deletions in the NK2 homeobox 1 gene (*NKX2-1*), located at 14q13.3, lead to symptoms associated with the brain, lungs, and thyroid, and the combination of these phenotypes is clinically recognized as the brain-lung-thyroid syndrome. Many types of nucleotide variants of *NKX2-1* have been identified, and phenotypic variability has been reported. Chromosomal deletions involving *NKX2-1* have also been reported; however, phenotypic differences between patients with nucleotide variants of *NKX2-1* and patients with chromosomal deletions involving *NKX2-1* have not been well established. Recently, we identified seven patients with 14q13 microdeletions involving the *NKX2-1*. Most patients exhibited developmental delay. This inquiry arises regarding the potential existence of haploinsufficiency effects beyond those attributed to *NKX2-1* within the 14q13 microdeletion. However, a literature review has shown that developmental delay is not rare in patients with nucleotide alterations in *NKX2-1*. Rather, motor function impairment may have affected the total developmental assessment, and the haploinsufficiency of genes contiguous to *NKX2-1* is unlikely to contribute to developmental delay.

Keywords Brain-lung-thyroid syndrome, chromosomal microarray testing, movement disorder, language delay

1. Introduction

The NK2 homeobox 1 gene (*NKX2-1*; MIM* 600635), located on chromosome 14 long arm 13.3, encodes a protein initially identified as thyroid-specific transcription factor-1 (TTF-1) (*1*). This gene is highly expressed in the thyroid, lungs, and pituitary glands (*https://www.proteinatlas.org/*), and murine knockout studies have established its critical role during the embryogenesis of those organs (*2*). In 1998, Devriendt *et al.* first reported the case of an infant with neonatal thyroid dysfunction associated with an *NKX2-1* deletion (*3*). Subsequently, monoallelic pathogenic variants and heterozygous deletions of *NKX2-1* have been identified to be associated with a complex phenotype involving choreoathetosis, respiratory problems, and hypothyroidism (*4*), constituting the triad of brain-lung-thyroid syndrome (BLTS) (*5*). Patients with BLTS typically experience respiratory failure during the neonatal period (*6*). In early infancy, a diagnostic evaluation conducted due to recurrent upper respiratory infections and stunted growth typically reveals the presence of subclinical hypothyroidism. Then, mildly delayed acquisition of motor milestones with involuntary movements including ataxia and chorea are gradually observed. Hence, these motor disorders are acknowledged as the primary manifestation of brain involvement in BLTS (*7*).

Recently, we identified seven new patients with 14q13 microdeletions including *NKX2-1*. Generally, patients with chromosomal microdeletions exhibit clinical phenotypes involving multiple contiguous genes owing to the haploinsufficiency effect of the deletion. Therefore, it is important to elucidate whether haploinsufficiency affects genes other than *NKX2-1* that are present within the 14q13 microdeletion region.

Herein, we discuss the phenotypic differences between microdeletions including *NKX2-1* and intragenic variants within *NKX2-1*.

2. Patients and Methods

This study was performed in accordance with the Declaration of Helsinki, and requisite permission was obtained from the ethics committee of Tokyo Women's Medical University. Peripheral blood samples were drawn from patients after obtaining written informed consent from their parents. Genomic DNA was extracted from the blood samples using a QIAamp DNA extraction kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Chromosomal microarray analysis (CMA) was performed using an Agilent Microarray 60 K kit (Agilent Technologies, Santa Clara, CA, USA) as previously described (*8*). Aberrations in the genomic copy number were visualized using the Agilent Genomic Workbench version 7 (Agilent Technologies). In this study, all genomic coordinates are referred to as GRCh37/hg19.

The clinical information of patients with 14q13 microdeletions was obtained from their attending physicians. Genotype-phenotype correlation was analyzed for patients with 14q13 microdeletions, in which previously reported patients were also included. The patients who showed microdeletions within chr14:33,000,000–41,000,000 were included in this analysis.

3. Results

3.1. Microdeletions

Five different microdeletions involving *NKX2-1* were detected in the seven patients (Figure 1). One of the microdeletions was detected in a sibling (an older sister and a male-female twin; patients 5–7). The genotypes and phenotypes of the patients are summarized in Table 1. In the literature, we identified sixteen previously reported patients, whose detailed information was available and who had microdeletions within chr14:33,000,000–41,000,000. These patients were included in Figure 1 and Table 1 for comparison with the patients in this study (*9-15*).

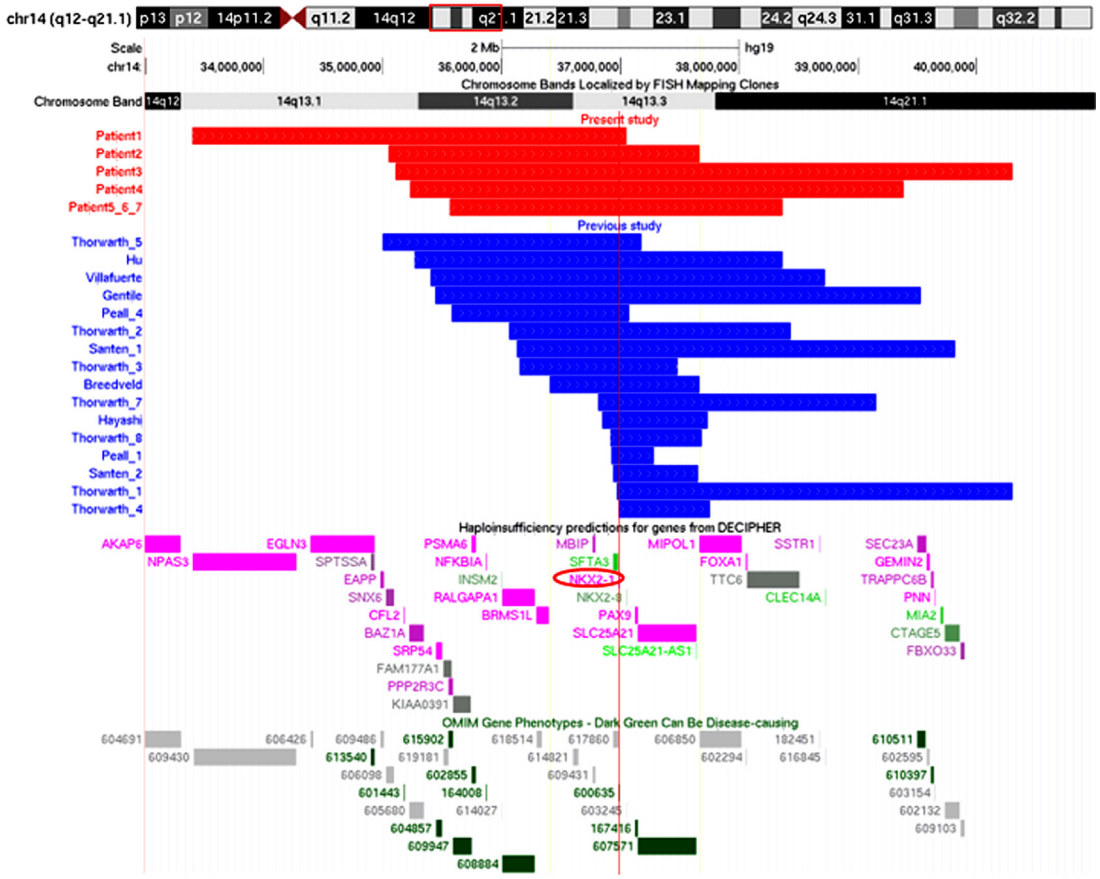

Figure 1. Genome map around 14q13 depicted by identified deletions. The map was captured from the UCSC genome browser (*https://genome.ucsc.edu/*). Regions of the identified deletions are depicted by custom tracks with red and blue bars (red for the deletions identified in this study, and blue for previously reported deletions). For haploinsufficiency prediction track, genes with magenta shades indicate a higher expectation of being haploinsufficient and genes with green shades indicate a lower expectation of being haploinsufficient. For OMIM gene phenotypes track, dark green and light gray indicate whether the genes are associated with OMIM phenotype or not, respectively. *NKX2-1* is highlighted by a red circle and the its location is shown by a red dotted line. All data are converted to GRCh37/hg19.

Table 1. Summary of the genotype and phenotype of the patients with 14q13 microdeletions

	This study							Santen *et al.* (2012)		Hu *et al.* (2013)	Peall *et al.* (2013)	
	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 1	Patient 2		Patient 1	Patient 4
Gender	Male	Male	Female	Male	Female	Male	Female	Female	Male	Female	Female	Male
Age	9y5m	5y6m	3y1m	17y	8y	3y	3y	7y	5y	15y	11y	6y
Deletion region*												
Start	33,399,986	35,050,201	35,110,121	35,231,236	35,564,225	35,564,225	35,564,225	36,129,971	36,940,484	35,268,524	36,924,171	35,581,654
End	37,051,206	37,666,592	40,301,821	39,387,699	38,366,977	38,366,977	38,366,977	39,816,401	37,657,846	38,367,321	37,283,221	40,301,792
Deletion size (Mb)	3.7	2.6	5.2	4.2	2.8	2.8	2.8	3.7	0.7	3.1	0.4	4.7
Gestational age (W)	38	38	40	37	41	37	37	42	NA	NA	NA	NA
Birth weight (g)	3,008	2,928	3,182	3,278	3,748	2,550	2,631	3,600	NA	NA	NA	NA
Short stature	+	+	-	+	+	+	+	-	-	+	NA	NA
Neonatal respiratory distress	-	+	+	+	-	+	+	-	+	-	-	-
Hypothyroidism	-	+	+	-	+	+	+	+	+	+	-	+
Involuntary movements including ataxia	-	+	+	+	+	-	-	+	+	+	+	+
Developmental delay	Moderate	Border	Mild	Moderate	Moderate	Severe	Severe	Mild	-	+	+	+
Other finding	Undescended testis	Pulmonary artery bifurcation stenosis and distinctive facial findings			Anodontia							

	Thorwarth *et al.* (2014)							Hayashi *et al.* (2015)		Gentile *et al.* (2016)	Villafuerte *et al.* (2018)	Positive ratio
	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 7	Patient 8	II-1	II-2			
Gender	Male	Male	Female	Male	Female	Male	Female	Famale	Famale	Female	Female	
Age	NA	NA	NA	NA	NA	NA	NA	14y	7y	15m	10y	
Deletion region*												
Start	36,972,991	36,068,376	36,151,329	36,988,681	35,001,682	36,814,106	36,919,999	36,852,260	36,852,260	35,443,407	35,404,289	
End	37,072,789	38,433,164	37,483,407	37,755,695	37,176,663	39,156,509	37,684,890	37,735,951	37,735,951	39,527,387	38,723,530	
Deletion size (Mb)	1.0	2.4	1.3	0.8	2.2	2.3	0.8	0.9	0.9	4.1	3.3	
Gestational age (W)	NA	NA	NA	NA	NA	NA	NA	NA	40	39	NA	
Birth weight (g)	NA	NA	NA	NA	NA	NA	NA	NA	NA	2620	NA	
Short stature	NA	NA	NA	NA	NA	NA	NA	NA	NA	-	-	
Neonatal respiratory distress	+	-	-	-	-	+	+	+	+	-	+	52%
Hypothyroidism	+	+	+	+	+	+	+	+	+	+	+	87%
Involuntary movements including ataxia	+	+	-	+	+	+	+	+	+	-	+	78%
Developmental delay	NA	NA	NA	NA	NA	NA	NA	+	+	-	+	
Other finding										Craniofacial findings	Craniofacial findings	

*, genomic positions referred to GRCh37/hg19 (All data are unified to the same build). y, years; m, months; Mb, megabase; W, weeks. Clinical information of the case reported by Breedveld *et al.* (7) is unavailable and not included in this table.

3.2. Patient reports

Clinical features of the patients are summarized in Table 1.

Patient 1, a 9-year-5-month-old boy, was born at 38 weeks of gestation without asphyxia. His birth weight was 3008 g. The right undescended testis was observed. He showed generalized hypotonia and psychomotor developmental delay since infancy, walking alone at three years of age. At present, his height is 118.6 cm (-2.5 SD), weight is 21 kg (-2.3 SD), and occipitofrontal circumference (OFC) is 51.5 cm (-0.7 SD), indicating short stature. He has developed established patterns for toilet habits and wearing clothing. Although he could walk by himself, he fell easily because of his ataxic gait. Thus, he required a handrail when going up and down the stairs. His intellectual quotient (IQ) was 33, indicating a moderate intellectual disability.

Patient 2 is a 5-year-6-month-old boy, who was born at 38 weeks of gestation without asphyxia. His birth weight was 2,928 g. Due to transient neonatal hypercapnia, he required transient ventilatory management. Pulmonary artery bifurcation stenosis was also observed. He showed distinctive facial features, including frontal bossing, epicanthus, long philtrum, large ear cups, and a congenital ear fistula. Although the patient showed elevated levels of thyroid-stimulating hormone, no medication was prescribed. His development was mildly delayed, with laughter noted at three months, head control at five months, turning over at six months, crawling and sitting at 12 months, and standing with support at 18 months. He uttered a two-word sentence after two years. At present, his height is 98.8 cm (-2.4 SD), weight is 21 kg (-2.3 SD), and OFC is 51.5 cm (-0.7 SD), indicating short stature. He has established his activities in daily life. His running and jumping movements were ataxic; however, dysarthria has been noted. The patient's IQ was 75.

Patient 3, a 3-year-1-month-old girl, was born at 40 weeks of gestation without asphyxia. The patient's birth weight was 3,182 g. After birth, she showed temporary respiratory distress but no feeding problems. Due to hypothyroidism, levothyroxine treatment was initiated on day eight. There was a history of viral infection at 2 months. She showed a mild motor developmental delay, with head control at 3 months, turning over at 7 months, sitting at 12 months, crawling and standing with support at 13 months, and walking alone at 15 months of age. However, her language development was not delayed with three-word sentences spoken at three years. Her developmental quotient was 60, indicating a mild delay. She shows gait instability and athetosis, although she can climb stairs using a handrail. At present, her height is 89.4 cm (-0.7 SD), weight is 13.5 kg (+0.2 SD), and OFC is 47.8 cm (-0.4 SD).

Patient 4 is a 17-year-old boy, who was born at 37 weeks of gestation without asphyxia. His birth weight was 3,278 g. The patient experienced transient neonatal hyperpnea associated with pulmonary hypertension. The patient had a history of recurrent pneumonia accompanied by bronchomalacia and gastroesophageal reflux. His motor development was severely delayed, with head control at 2 years, sitting at 6 years, and standing with support at 10 years. However, his total IQ score was 46, indicating a moderate intellectual disability. At present, his height is 146.2 cm (-4.2 SD), weight is 36.6 kg (-2.5 SD), and OFC is 52.5 cm, (+0.3 SD), indicating short stature. His daily life skills have not yet been fully established. The patient still shows an ataxic gait and falls easily.

Patients 5, 6, and 7 are siblings. Interview revealed that their mother had shown developmental delay during childhood. The mother exhibits a short stature. Due to hypothyroidism, she has continued to take levothyroxine. The father of patient 5 and the father of patients 6 and 7 are different. Patient 5 is a 9-year-old girl born as a result of her mother's second pregnancy. The patient was born at 41 weeks of gestation with a birth weight of 3,748 g. She showed developmental delay with head control at 10 months, walking with support at 16 months, and use of simple words at 12 months. The patient experienced recurrent pneumonia during early childhood. Levothyroxine was prescribed for hypothyroidism. She also had short stature. Involuntary movement and dysarthria were also observed. When necessary, patients used a wheelchair. She also had anodontia in some teeth.

Patients 6 and 7 are 3-year-old male and female twins, respectively. They were born at 37 weeks of gestation with birth weights of 2,550 g and 2,631 g, respectively. Both patients needed respiratory management at the NICU due to neonatal respiratory failure. Nitric oxide inhalation was administered to patient 6 (first twin) for persistent pulmonary hypertension. Surfactant administration was required for patient 7 (second twin) because of associated pulmonary hemorrhage. After extubation, both patients experienced difficulty in weaning from oxygen and continued home oxygen therapy. Both patients showed developmental delay, turning over at 18 months of age without sitting. They also exhibited hypotonia and short stature. Due to hypothyroidism, levothyroxine was prescribed to both patients.

4. Discussion

The classical triad of BLTS is not always present, and only 50% of patients with *NKX2-1* involvement develop the complete triad (*16*). The severity of the phenotypes also varies, even within the same family (*17*). As shown in Table 1, not all patients identified in this study fulfilled the triad.

Movement disorders, including choreoathetosis, are thought to be the main neurological symptoms

associated with *NKX2-1*–related abnormalities. However, most patients in this study showed mild-to-severe developmental delay (Table 1). Thus, we hypothesized that developmental delay may be a symptom specific to microdeletions and may be attributed to haploinsufficiency affecting another gene within the deletion.

Santen *et al*. reported seven patients with 14q deletions (*9*). Two of them showed microdeletions including *NKX2-1*, and one of the two patients showed mild developmental delay (Table 1). Hamvas *et al*. reported five patients with *NKX2-1* deletions and 16 patients with *NKX2-1* variants (*18*). All patients with *NKX2-1* deletions exhibited developmental delay in association with language delay or behavior problems. On the other hand, only three of 16 patients with *NKX2-1* variants showed language delay. The "Patient 2", reported by Shetty *et al*. (*19*), demonstrated 14q13-q21.1 microdeletion; however, details are unavailable. The patient was diagnosed as having autism; whereas, such clinical features are considered as a nonmotor neurological manifestations of *NKX2-1* abnormalities. Thorwarth *et al.* (*12*) reported 32 patients with *NKX2-1* involvement. Eleven patients had microdeletions including *NKX2-1*. Twelve patients showed low-normal levels of IQ (*12*); however, whether they had microdeletions or nucleotide variants in the gene remains unknown. Peall *et al.* (*11*) reported ten patients with *NKX2-1*–related abnormalities. Two of ten patients exhibited microdeletions involving *NKX2-1*, and exhibited developmental delay. In contrast, developmental delay was also observed in four of the eight patients with *NKX2-1* intragenic nucleotide variants (50%). Parnes *et al*. reported five patients with *NKX2-1* variants. Four of five patients show speech delay together with motor delay (*20*). From these findings, we concluded that the comorbidity rate of developmental delay does not change significantly depending on whether patients had microdeletions involving *NKX2-1* or nucleotide variants of *NKX2-1*.

As shown in Figure 1, in the neighboring region of *NKX2-1*, there are some genes with a higher expectation of being haploinsufficient. Although some of them are related to OMIM phenotypes, most of them are related to unknown inheritance pattern. Previously, haploinsufficiency of the Ral GTPase activating protein alpha subunit 1 gene (*RALGAPA1*) was considered to be related to developmental delay and epilepsy (*21*), and *RALGAPA1* was included in the commonly deleted region in this study (Figure 1). However, *RALGAPA1* was later identified as a gene related to developmental epileptic encephalopathy associated with an autosomal recessive trait (*22*), and carriers of loss-of-function variants of *RALGAPA1* showed no symptoms. Hence, haploinsufficiency of *RALGAPA1* was not related to clinical symptoms, such as the developmental delay observed in patients with 14q13 microdeletions. Because *RALGAPA1* is the only gene highly expressed in the brain in the contiguous region of *NKX2-1*, the haploinsufficiency of other genes contiguous to *NKX2-1* is unlikely to contribute to developmental delay.

In conclusion, alteration of *NKX2-1* itself would contribute to developmental delay in patients.

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**Address correspondence to:*
Toshiyuki Yamamoto, Division of Gene Medicine, Graduate School of Medicine, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku-ward, Tokyo 162-8666, Japan.
E-mail: yamamoto.toshiyuki@twmu.ac.jp

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Original Article

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Role of IFITM2 in osteogenic differentiation of C3H10T1/2 mesenchymal stem cells

Yongtao Zhang[1], Xiangdong Li[2], Shanshan Zhang[1], Junfeng Li[1], Meilin Liu[1], Yanqin Lu[1,3,*], Jinxiang Han[1,3,*]

[1] 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;
[2] Department of Clinical Laboratory, The First Affiliated Hospital of Shandong First Medical University & Shandong Provincial Qianfoshan Hospital, Ji'nan, Shandong, China;
[3] Department of Endocrinology and Metabology, The First Affiliated Hospital of Shandong First Medical University & Shandong Provincial Qianfoshan Hospital, Ji'nan, Shandong, China.

SUMMARY Interferon-inducible transmembrane (IFITM) are a family of small proteins localized to plasma and endolysosomal membranes. Their functions beyond restricting viral entry and replication have been revealed in recent years. IFITM5 is involved in bone mineralization and is an osteogenic cell surface marker. IFITM1 and 3 interact with desmin and myosin, and are involved in myogenic differentiation. This study found upregulation of *Ifitm2* during osteogenic differentiation of C3H10T1/2 cells. This positively correlated to the expression of osteogenic differentiation markers *Col1a1, Alp, Runx2*, and *Ocn*. Knockdown of *Ifitm2* by siRNAs inhibited osteogenic differentiation, calcium deposition, and osteogenic marker expression of C3H10T1/2 cells. The osteoblast transcriptome revealed that knocking down *Ifitm2* affected the expression Wnt signaling pathway-related genes, including Wnt family members, their receptors Lrp, Frizzled, and Lgr, and transmembrane molecule Rnf43 that suppresses the Wnt signaling pathway. Luciferase assays indicated enhancement of canonical Wnt signaling pathways by *Ifitm2* overexpression. Furthermore, IFITM2 was colocalized in the metaphyseal bone and growth plate of the mouse tibial bone with SP7, a transcription factor essential for osteoblast differentiation and bone formation. These findings reveal a possible novel function and potential mechanisms of *Ifitm2* in osteogenic differentiation.

Keywords IFITM2, osteogenic differentiation, Wnt/β-catenin signaling pathway, TOP/FOP assay, C3H10T1/2 cells

1. Introduction

Mesenchymal stem cells can differentiate into mature osteoblasts lining the bone surface and osteocytes embedded in bone (*1*). Osteogenesis is a three-step process consisting of the proliferative phase, matrix maturation, and mineralization (*2*). The process is characterized by distinctive sequentially expressed osteoblast markers, including alkaline phosphatase (ALP), type I collagen (Col1), osteopontin (OPN), bone sialoprotein (BSP), and osteocalcin (OCN) (*3-7*). The effects of interferon (IFN) on differentiation of osteoblastic cells have recently drawn attention (*8,9*). Even without exogenous IFN and virus infection, in a cell model of osteogenic differentiation, the cells spontaneously produce endogenous IFN that increases expression of interferon-stimulated genes, including interferon-inducible transmembrane (IFITM) proteins (*10*).

IFITM proteins belong to the small interferon-stimulated family with molecular masses ranging from 10 to 20 kDa (*11*). The family members include IFITM1, IFITM2, IFITM3, IFITM5, and IFITM10 in humans, and IFITM1, IFITM2, IFITM3, IFITM5, IFITM6, IFITM7 and IFITM10 in mice (*12,13*). Except for IFITM3, IFITMs are highly expressed in rat bone marrow (*14*). IFITM1–3 play a vital role in blocking viral infection (*15,16*). IFITMs are increasingly thought to have a role in cancer and innate immunity (*17*). We previously found upregulation of IFITM1–3 during myogenic differentiation, and IFITM1 and 3 interact with desmin and myosin (*18*). Hence, the functions of IFITMs

extend to multiple lineages of differentiation. The study investigated *Ifitm2* expression during osteogenic differentiation and the related signaling pathways.

2. Materials and Methods

2.1. Cell culture and osteogenic differentiation of C3H10T1/2 cells

C3H10T1/2 cells were cultured in high-glucose Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C with 5% CO_2. Osteogenic differentiation of C3H10T1/2 cells was performed in 6- or 24-well plates. At 24 h post-seeding, cells were cultured in osteogenic differentiation medium containing 10 mM β-glycerophosphate, 100 nM dexamethasone, and 0.2 mM ascorbic acid. C3H10T1/2 cells were osteogenically stimulated for 14 days with medium exchanges every 3 days. The osteogenic differentiation capacity was determined by alizarin red S staining.

2.2. Alizarin red S staining

C3H10T1/2 cells were washed with PBS and fixed with 4% paraformaldehyde at room temperature for 15 min. After rinsing with PBS twice, the cells were stained with a 0.2% alizarin red S solution at room temperature for 20 min and then washed with deionized water until the supernatant was colorless. Stained C3H10T1/2 cells were imaged using a digital camera (Canon, Japan). After imaging, 10% cetylpyridinium chloride (Sangon Biotech, China) was applied in the dark for 30 min, and then 100 μL was transferred to a 96-well plate to measure the OD value at 560 nm. Analyses were performed in at least three independent experiments. Absorbance at day 0 was used to normalize the alizarin red S staining results.

2.3. RNA extraction and RT-qPCR

Total RNA was isolated from C3H10T1/2 cells cultured in differentiation or normal media using a 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 digestion with DNase I (Takara, Japan), 1 μg total RNA was reverse transcribed to cDNA using a ReverTra Ace qPCR RT Kit (Takara, Japan). Quantitative PCR was performed using 2× SYBR Green qPCR Mix (SparkJade, Bio, China) and a LightCycler 480. PCR conditions were as follows: initial 5 min denaturation at 95 ℃, followed by 45 cycles of amplification at 95 ℃ for 10 sec, 60 ℃ for 10 sec and 72 ℃ for 15 sec. To quantify the expression of each gene of interest, mRNA expression levels were normalized to the mRNA level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Relative gene expression was calculated with the $2^{-\Delta\Delta Ct}$ method. Each sample was analyzed in triplicate. Primer sequences were as follows: *Gapdh*: 5′-CATCCCAGAGCTGAACG-3′ (forward), 5′-CTGGTCCTCAGTGTAGCC-3′ (reverse); *Col1a1*: 5′-GCTCCTCTTAGGGGCCACT-3′ (forward), 5′-ATTGGGGACCCTTAGGCCAT-3′ (reverse); *Alp*: 5′- GCCCGGCCGAGTACA-3′ (forward), 5′-CTGGCCAGAAACTTCACCTT-3′ (reverse); *Runx2*: 5′-ACAGAGCTATTAAAGTGACAGTGGAC-3′ (forward), 5′- GGCGATCAGAGAACAAACTAGG 3′ (reverse); *Ocn*: 5′-GCGCTCTGTCTCTCTGACCT-3′ (forward), 5′-TTCAGGAGGGTAGTTACCCAAA-3′ (reverse).

2.4. Western blotting

C3H10T1/2 cells in a 6-well plate were washed with cold PBS. Then, the cells were lysed in 200 μL RIPA lysis buffer with 1% PMSF (Cwbio, China) at 4°C. The extracted proteins were quantified using a BCA protein concentration assay kit (Biosharp, China). Protein samples (20 μg/lane) were resolved by 12% SDS-PAGE. Proteins were then transferred to a polyvinylidene fluoride membrane (0.45 μm, Biosharp). The membrane was blocked with 5% dry skim milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 2 h at room temperature.

The membrane was incubated with a primary antibody against COL1A1 (1:2000, 67288-1-Ig, Proteintech, USA), ALP (1:2500, PA5-63148, Thermo Fisher, USA), RUNX2 (1:1000, 82636-2-RR, Proteintech), OCN (1:500, 3418-1-AP, Proteintech), IFITM2 (1:1000, bs-15517R, Bioss, China), or GAPDH (1:3000, ab8245, Abcam, USA) overnight at 4°C. After washing with 0.1% Tween in PBS, the membrane was incubated with an anti-rabbit-horseradish peroxidase-conjugated secondary antibody (1:2000, SA00001, Proteintech) for 1 h at 37°C. A Super-sensitive ECL chemiluminescent substrate kit (Biosharp, China) was used to develop protein bands. ImageJ-win64 (Rawak Software Inc., Stuttgart, Germany) was used for densitometry.

2.5. siRNA transfection

Small interfering RNA based on our previous study (*18*) and directed against mouse *Ifitm2* was synthesized by GenePharma (Shanghai, China). Before siRNA transfection, C3H10T1/2 cells were seeded at 2×10^5 cells/well in 6-well plates in serum and antibiotic-free DMEM. Transfections were performed with siRNA-*Ifitm2* or negative control siRNA (si-NC) using ExFect transfection regent (Vazyme, China) in accordance with the manufacturer's instructions. After 6 h of transfection, the medium was replaced with osteogenic differentiation medium. After 5 days, proteins were extracted for

western blotting, and total RNA was extracted for RNA-sequencing analysis.

2.6. RNA-seq and bioinformatics analysis

Total RNA was extracted using an RNeasy Mini Kit (QIAGEN, China), following the manufacturer's instructions. Two micrograms of RNA were used as the input material for the RNA sample preparation. Sequencing libraries were generated using a NEBNext Ultra RNA Library Prep Kit for Illumina (#E7530L, NEB, USA), following the manufacturer's recommendations. Index codes were added to attribute sequences to each sample. The libraries were sequenced using a NovaSeq 6000 (pair-end 150 bp). All reads were mapped to the mouse reference genome using HISAT2 (v.2.2.1) as reported previously (*19*). RNA counts were generated by *featureCounts* (v.2.0.0) (*20*). Differential expression analysis was implemented using the edgeR package (v.3.34.1) as reported previously (*21*). Genes with an expression fold change of ≥ 1.5 or ≤ 1.5 and adjusted P-value of < 0.05 were identified as significantly differentially expressed genes. For further analysis, a volcano plot was generated by the ggplot2 R package. Heat maps were generated by the pheatmap R package.

Cluster and pathway analyses were performed using the KEGG pathway database (*http://www.kegg.jp/kegg/*) and DAVID bioinformatics resources 6.8 (*https://david.ncifcrf.gov/*). Enriched pathways were ranked using the combined score calculated by the software. To perform gene ontology (GO) analysis, data of the function annotation diagram were obtained using the DAVID website, and data with $P < 0.05$ were selected. Data processing and mapping were performed using R-project (v4.0.5) and Rstudio software (v1.3.1093).

2.7. TOP/FOP flash luciferase reporter assay

HEK293T cells were seeded into a 96-well plate at 1×10^4 cells per well. Then, 250 ng TOP/FOP, 50 ng pTK-RL plasmid, and 100 ng pMCV6-Ifitm2 overexpression vector or control were transiently cotransfected into the cells using ExFect Transfection Reagent (Vazyme). The activities of firefly and Renilla luciferase reporters were measured in triplicate at 48 h post-transfection using the Duo-Lite Luciferase Assay System (Vazyme) in accordance with the manufacturer's instructions. Firefly luciferase activity was normalized to the Renilla luciferase activity. The TOP/FOP ratio was used to indicate β-catenin-driven transcription.

2.8. Immunofluorescence staining

C3H10T1/2 cells were fixed with 4% paraformaldehyde in phosphate-buffered saline, permeabilized with 0.25% Triton X-100, and blocked in a blocking solution (2% glycine, 2% bovine serum albumin, 5% fetal bovine serum, and 50 mM NH_4Cl in phosphate-buffered saline) for 1 h. Then, the cells were incubated with primary antibodies for 12 h at 4℃ and then with corresponding secondary antibodies conjugated to various fluorescent dyes. For nuclear staining, the cells were incubated with 1 μg/mL DAPI (Sigma, USA) for 10 min at room temperature. After washing with PBS, the cells were subjected to microscopy.

2.9. Double immunofluorescence staining of paraffin-embedded sections

The animal study was approved by ethics committee of Shandong First Medical University & Shandong Academy of Medical Sciences, and was performed in accordance with ethical standards stated in the 1964 Declaration of Helsinki and its later amendments. The tibia was harvested from 15-week-old male mice. Specimens were immersed in paraffin and then sectioned. Double immunofluorescence staining was performed on 5-μm-thick paraffin-embedded sections of formalin-fixed mouse tibial samples. Samples were deparaffinized and rehydrated. After blocking with normal goat serum for 30 min at room temperature, a rabbit anti-IFITM2 antibody (1:500, bs-15517R, Bioss) was applied at 4°C overnight, followed by an Alexa 488-conjugated goat anti-rabbit IgG secondary antibody (1:500, A-11001, Thermo Fisher) for 1 h at room temperature. Additional immunofluorescence staining was then performed by incubation with an anti-SP7 primary antibody (1:500, Ag29889, Proteintech), for 1 h at room temperature, followed by incubation with an Alexa Fluor 594-conjugated fluorescent goat anti-mouse IgG secondary antibody (1:100, A-11005, Thermo Fisher) for 1 h at room temperature. After washing with PBS, 10 μg/mL DAPI was applied in the dark for 10 min. Samples were sealed with an anti-fluorescence attenuation sealing sheet. Images were obtained under a fluorescence microscope.

2.10. Statistical analysis

Results are presented as the mean ± standard error of the mean. To identify significant differences, statistical comparisons were made by one-way ANOVA and the Tukey multiple comparisons test using GraphPad Prism software (v.8.0) (GraphPad Software Inc, USA). $P < 0.05$ was considered statistically significant. All experiments were repeated at least three times.

3. Results

3.1. *Ifitm2* is upregulated during osteogenic differentiation of C3H10T1/2 cells

Calcium deposition of C3H10T1/2 cells during osteogenic differentiation was detected by alizarin red S staining. After 5, 7, 10, and 14 days, the area of red

staining in the surrounding material was increased gradually, indicating enhanced precipitation of calcium salts as induction time increased (Figure 1A). Quantitative analysis of osteogenic mineral deposition indicated a significant difference between the osteogenic differentiation group at day 5 and later days compared with day 0, which was uninduced (Figure 1B). Analysis of osteogenic differentiation markers was performed at 0–14 days of incubation in osteogenic differentiation medium. mRNA and protein expression levels of *Col1a1*, *Alp*, *Runx2*, and *Ocn* at days 10 and 14 after osteogenic differentiation induction was consistent and significantly increased ($P < 0.01$) (Figure 1C, D).

RT-qPCR analysis indicated that C3H10T1/2 cells in osteogenic induction medium at day 7 showed the highest expression of *Ifitm2*, but no significant increase was found at days 3 and 5 (Figure 2A). Overall trends showed an increase in IFITM2 protein expression during osteogenic differentiation of C3H10T1/2 cells with higher expression on days 3 and 5 compared with day 0 before induction and slightly decreased expression at day 7 ($P < 0.01$) (Figure 2B). Immunofluorescence also showed an increase in IFITM2 expression after 5 days of osteogenic induction compared with day 0 ($P < 0.01$) (Figure 2C).

3.2. siRNA downregulates *Ifitm2* expression during osteogenic differentiation of C3H10T1/2 cells

We hypothesized that *Ifitm2* plays a role in calcium homeostasis during osteogenic differentiation of C3H10T1/2 cells. Therefore, we silenced *Ifitm2* gene expression to examine its effect on mineralization of C3H10T1/2 cells during osteogenic differentiation (Supplement Figure S1, *http://www.irdrjournal.com/action/getSupplementalData.php?ID=176*). Alizarin red S staining showed reduced calcium deposition of osteogenically induced cells transfected with specific *Ifitm2* siRNAs ($P < 0.01$) (Figure 3A). Quantitative analysis showed that C3H10T1/2 cells with *Ifitm2* knockdown exhibited low calcium deposition at day 5 after osteogenic induction, whereas calcification was observed in si-NC and mock groups (Figure 3B). Regarding osteogenic marker genes, the expression levels of COL1A1, ALP, RUNX2, and OCN were significantly low in *Ifitm2* knockdown groups, which further indicated the involvement of *Ifitm2* in osteoblastic differentiation ($P < 0.01$) (Figure 3C, D).

3.3. RNA-seq and bioinformatics analysis

We used RNA-seq and bioinformatics analysis to assess gene expression changes triggered by *si-Ifitm2* transfection. A volcano plot of RNA-seq data revealed that 2,064 genes were upregulated (red) and 1,848 were downregulated (blue) after *Ifitm2* knockdown [fold change ≥ 1.5, false discovery rate (FDR) < 0.01] (Figure 4A). Heat map analysis revealed distinct genes after *Ifitm2* knockdown (Figure 4B, C; details on the genes are displayed in Supplementary Table S1, *http://www.irdrjournal.com/*

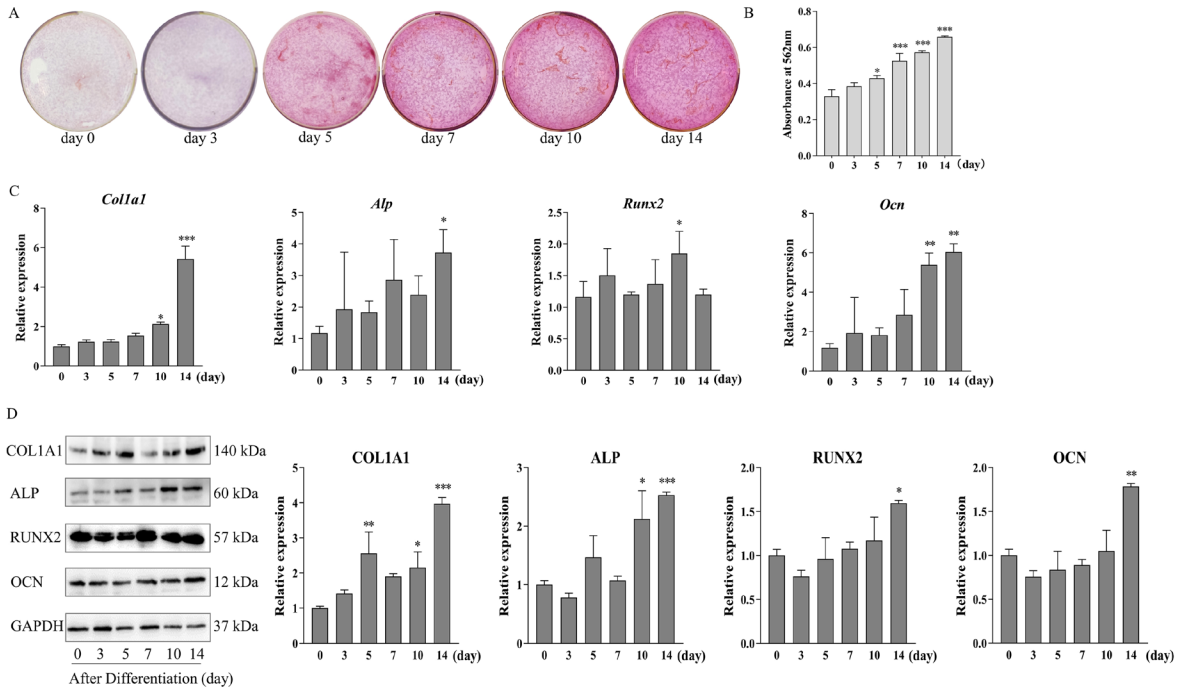

Figure 1. Alizarin red S staining and osteogenic biomarker expression of C3H10T1/2 cells at various days of osteogenic induction. (A) Representative alizarin red S staining of cells in 24-well plates. **(B)** Quantification of alizarin red S staining showed an increase in calcium deposition after osteogenic induction (**$P < 0.01$ *vs.* day 0 group). **(C)** Expression levels of *Col1a1*, *Alp*, *Runx2*, and *Ocn* measured by RT-qPCR. **(D)** Representative bands of COL1A1, ALP, RUNX2, OCN, and GAPDH in western blots. Semi-quantification of the band intensity in western blots is shown. Protein levels were normalized to GAPDH. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ *vs.* day 0 group.

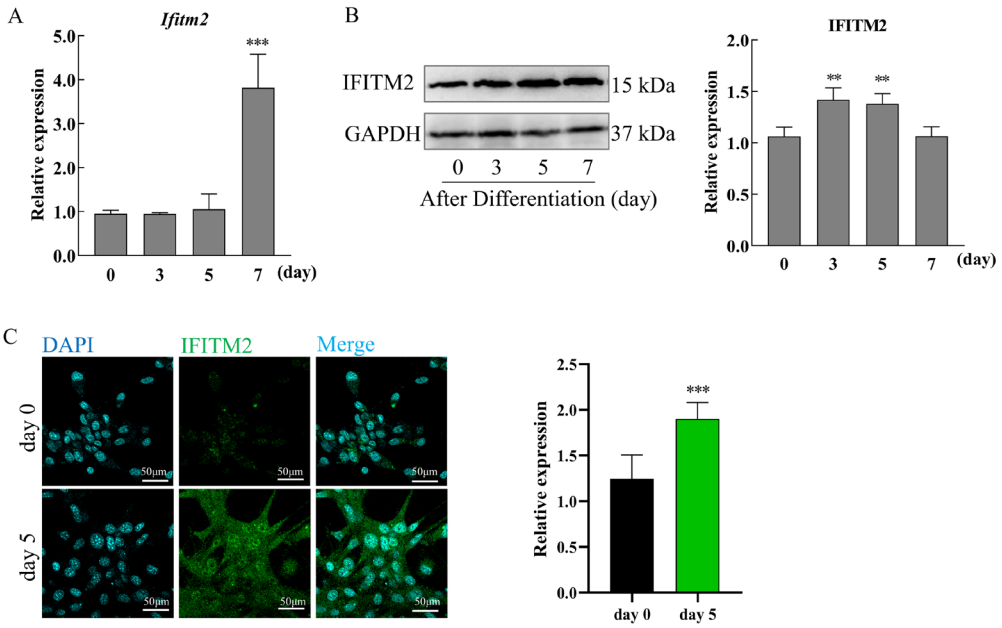

Figure 2. Increased expression of IFITM2 during osteoblastic differentiation of C3H10T1/2 cells. (A) Relative expression of *Ifitm2* mRNA measured by RT-qPCR after osteogenic induction for 3, 5, and 7 days. **(B)** Representative bands of IFITM2 in western blots and densitometry after osteogenic induction for 3, 5, and 7 days. Protein levels were normalized to GAPDH. **(C)** Immunofluorescence staining of IFITM2 (green) in C3H10T1/2 cells without or with osteogenic induction (day 5). Scale bars, 50 μm. Nuclei were visualized by DAPI staining. Quantitative analysis of the relative fluorescence intensity by one-way ANOVA is shown ($n = 3$). $^{**}P < 0.01$, and $^{***}P < 0.001$.

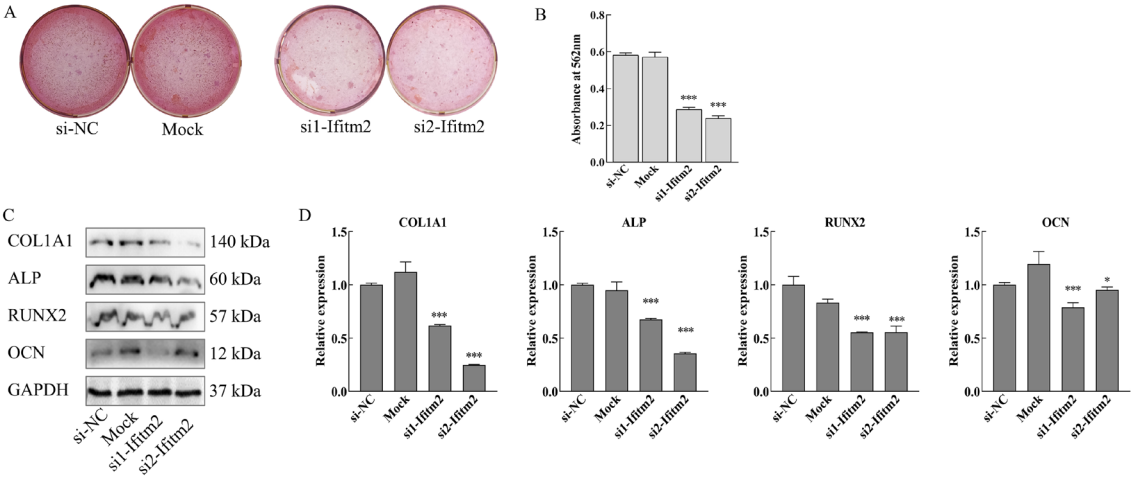

Figure 3. Alizarin red S staining and osteogenic biomarker expression of C3H10T1/2 cells transfected with si-*Ifitm2* after 5 days of osteogenic induction. (A) Alizarin red S staining indicated a decrease in calcium deposition in the si- *Ifitm2* group compared with siRNA negative control (si-NC) and transection reagent only (mock) groups. **(B)** Quantification of alizarin red S staining indicated that si-*Ifitm2* reduced the mineralization capacity from 49.19% to 41.89% compared with si-NC. **(C)** Representative bands of COL1A1, ALP, RUNX2, OCN, and GAPDH in western blots. **(D)** Quantification of the band intensities. Protein levels were normalized to GAPDH. Magnification: ×10. $^{*}P < 0.05$, $^{***}P < 0.001$.

action/getSupplementalData.php?ID=177). Clearly, *Ifitm2* knockdown by siRNAs changed the expression of genes involved in the Wnt signaling pathway, including Wnt receptors *Lgr4*, *Fzd1*, *Fzd3-7*, *Lrp8*, and *Lrp11*, *Wnt* family members *Wnt4*, *Wnt6*, *Wnt10a*, and *Wnt10b*, and *Znrf1* and *Rnf43* (Figure 4C).

GO analysis was performed to evaluate related biological processe (BP), cell components (CC) and molecular functions (MF) of the identified genes on the basis of their variability ranking. GO enrichment analyses revealed 1174 BP entries involving the ncRNA metabolic process, ncRNA processing, ribonucleoprotein complex biogenesis, muscle tissue development, tRNA metabolic process, ribosome biogenesis, negative regulation of cell cycle, 150 CC entries involving the transcription regulator complex, nuclear speck, preribosome, spindle, spliceosomal complex, nuclear envelope, and 149 MF entries involving the inositol phosphate phosphatase activity, histone deacetylase binding, protein kinase regulator activity, RNA methyltransferase activity,

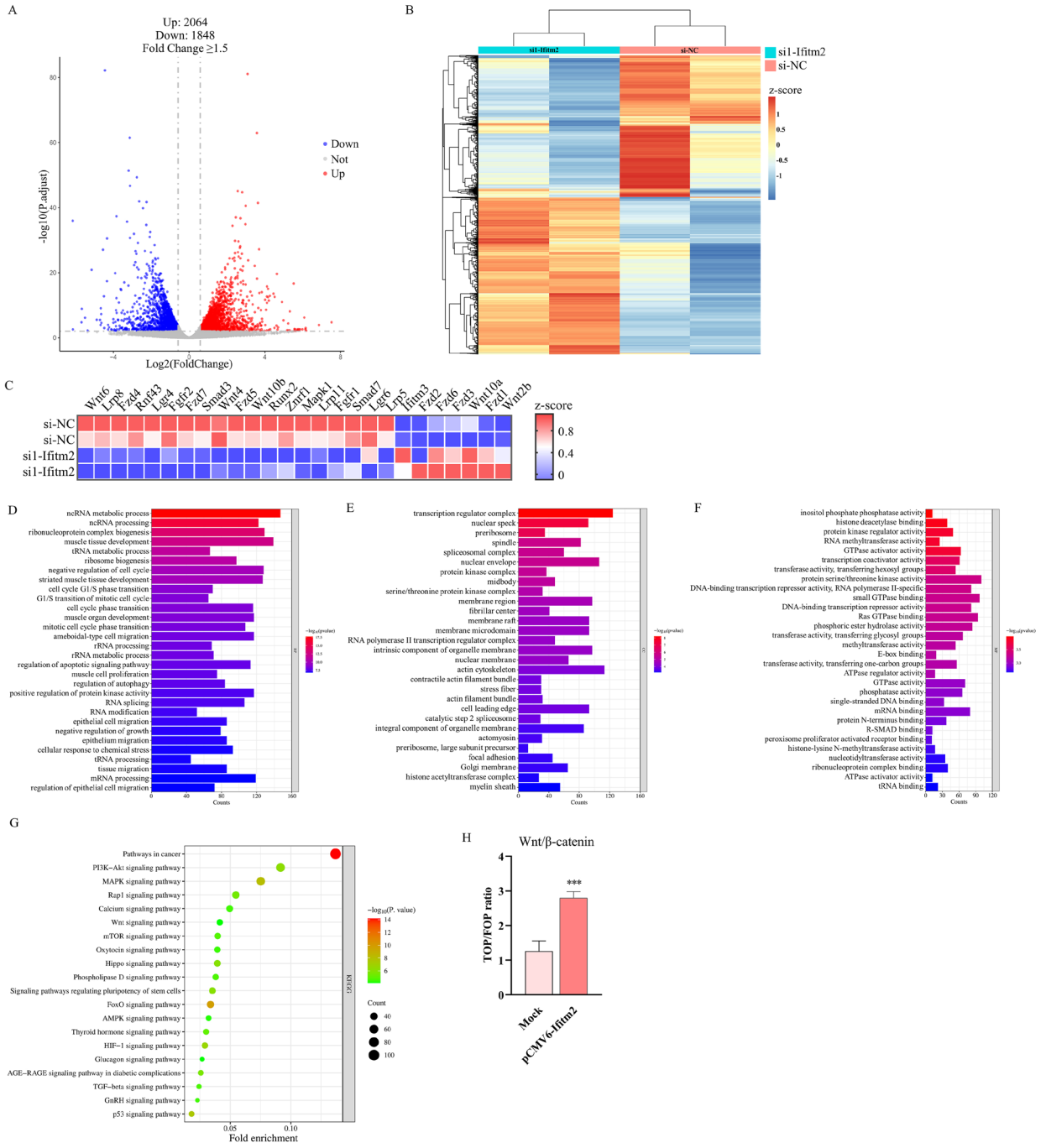

Figure 4. Volcano plot, heat map, GO, and KEGG analyses of RNA-sequencing data. **(A)** Volcano plots of significantly differentially expressed genes (FDR < 0.05 and |FC| ≥ 1.5; red, upregulated; blue, downregulated). **(B)** Heat map showing 3912 significantly (FDR < 0.05) differentially expressed genes between si1-*Ifitm2* and si-NC groups. Each row of the heat map represents the z-score transformed $\log_2$(1+FPKM) values of one differentially expressed gene across all samples (blue, low expression; red, high expression). **(C)** Significantly differentially expressed genes related to the Wnt signaling pathway. **(D–F)** Biological process (top 30), cellular components (top 30), molecular functions (top 30), and **(G)** KEGG enrichment analysis (top 20) of differentially expressed genes, respectively. **(H)** TOP/FOP luciferase reporter assays in HEK293T cells. TOP/FOP luciferase reporter activity was enhanced by *Ifitm2* overexpression.

GTPase activator activity, transcription coactivator activity (Figure 4D-F).

KEGG pathway analysis showed that the genes were mainly enriched in pathways in cancer, the PI3K-Akt signaling pathway, MAPK signaling pathway, Wnt signaling pathway, and signaling pathways regulating pluripotency of stem cells (Figure 4G). Among the dysregulated genes, we focused on the Wnt/β-catenin pathway and confirmed the results using a dual luciferase reporter assay. TOP/FOP luciferase reporter assays indicated that *Ifitm2* overexpression promoted luciferase activity to 2.2 fold (Figure 4H).

3.4. IFITM2 expression in mouse tibial tissue

To investigate the distribution of IFITM2 in bone-forming

tissue, we performed double immunofluorescence staining of mouse hind limb tissue sections to observe cells undergoing osteogenic differentiation. DAPI stains nuclei specifically (Figure 5A, E and I), IFITM2 were mainly detected in in growth plate cartilage and the trabecular bone area on the growth plate that has a high bone remodeling activity (Figure 5B, F and J). SP7 is a key transcriptional determinant of bone-secreting osteoblasts. It was expressed also in osteoblast on the surface of trabecular bone and cartilage (Figure 5C, E and K). Co-staining revealed that the entire tibia bone was positive for IFITM2 and SP7, which co-localized in the articular cartilage and metaphyseal bone (Figure 5D, H and L).

4. Discussion

Mesenchymal cells are ideal for stem cell-based therapy and have the capacity to differentiate into osteoblasts, chondrocytes, adipocytes, and myoblasts (*1,22*). Osteoblastogenesis is tightly regulated by complex cytokine networks under physiological and pathophysiological conditions (*2*). The IFITM family is well known for their functions in viral infection and innate immunity (*17,23-25*). IFITM5 is an osteoblast-specific membrane protein and functions as a positive regulatory factor for bone mineralization (*26,27*). IFITM5 affects Wnt signaling during tooth root development, although their interactions remain unclear (*28*). Wnt/β-catenin signaling is a major signaling pathway regulating skeletal mineralization (*29*). Downregulation of Wnt/β-catenin signaling by knockout of β-catenin in odontoblasts and cementoblasts inhibits tooth root development *in vivo* and *in vitro* (*30*). In our study, the expression of Wnt family members and their receptors, including *Lrp*, *fizzled*, and *Lgr*, was changed by downregulating *Ifitm2* expression. Moreover, expression of Rnf43, a transmembrane molecule that downregulates Wnt signaling, was decreased (*31*).

In the IFITM family, IFITM2 is a relatively newly evolved gene, and it is only present in *Homo sapiens*, *Gorilla gorilla*, and *Pan paniscus* of 26 primate species (*32*). Structurally, unlike IFITM1, which has an extended C-terminal, IFITM2 and IFITM3 have extended N-termini with an extra 20 or 21 amino acids (*33*). IFITM2 restricts entry of the CXCR4-tropic virus (*17*) and is positively associated with malignant gliomas. Enhanced *IFITM2* expression in glioblastomas predicts a malignant phenotype (*34*). In colon cancer, *IFITM2* is a novel p53-independent proapoptotic gene and highly expressed (*35*). Therefore, it is a potential therapeutic target for gastric and other cancers (*36*).

In this study, we found that *Ifitm2* was involved in osteogenic differentiation of C3H10T1/2 cells. It was upregulated during osteogenic differentiation, and knocking down *Ifitm2* led to downregulation of osteogenesis markers. Transcriptome profiling and TOP/FOP assays revealed the involvement of Wnt/β-catenin signaling induction in *Ifitm2* overexpression, which indicated a potential interaction of *Ifitm2* with the canonical Wnt signaling pathway contributing to osteogenic differentiation. However, how IFITM2

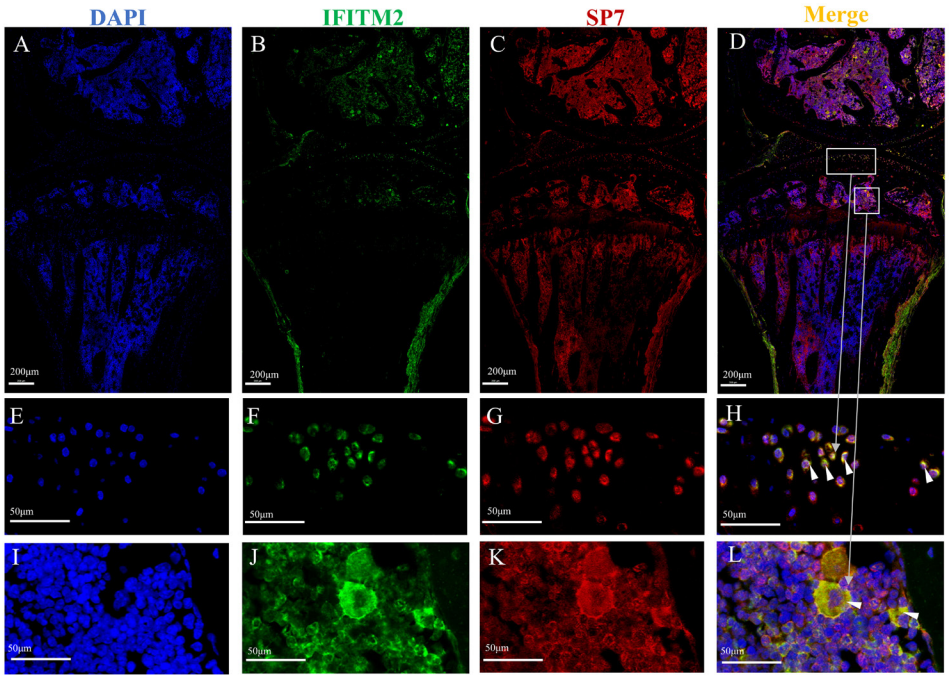

Figure 5. Expression of IFITM2 examined by histology in mice. (A–D) Representative immunofluorescence staining of IFITM2 (green) and SP7 (red) in the hind limb bone of 15-week-old mice. Scale bars, 200 μm. **(E–H)** Articular cartilage and **(I–L)** metaphyseal bone regions magnified to display positive cells. White arrow indicates IFITM2 and SP7 co-localization in endochondral ossification centers and articular cartilage. Scale bar: 50 μm. DAPI (blue) was used to counterstain nuclei.

interacts with the Wnt signaling pathway and whether it has similar mechanisms to IFITM5 in osteogenic differentiation remain unclear.

IFITM protein overexpression promotes interferon-β production (*37*). In early osteoblastic differentiation, IFN inhibits ECM synthesis, leading to delayed bone formation (*38*). Hence, we hypothesized that upregulated expression of IFITM2 during osteogenic differentiation may be independent of interferon and involve the canonical Wnt signaling pathway. A shortcoming of this study is that further experiments are needed to support an association between the canonical Wnt signaling pathway and overexpression or knockdown of *Ifitm2*, and we did not reveal the relationship between *Ifitm2* and Wnt the signaling pathway in detail.

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*Address correspondence to:
Yanqin Lu and Jinxiang Han, The First Affiliated Hospital of Shandong First Medical University & Shandong Provincial Qianfoshan Hospital, No. 16766 Jingshi Road, Ji'nan, Shandong 250013, China.
E-mail: yqlu@sdfmu.edu.cn (LYQ), jxhan@sdfmu.edu.cn (HJX)

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Original Article

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Surgical outcomes of locally advanced gastrointestinal stromal tumors after multivisceral resection: A retrospective study of 64 patients at a single institution

Hui Qiu[§], Zhen Wang[§], Bonan Liu, Rongze Sun, Xiuyun Tian, Chunyi Hao[*]

Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education, Beijing), Department of Hepato-Pancreato-Biliary Surgery, Sarcoma Center, Peking University Cancer Hospital and Institute, Beijing, China.

SUMMARY To analyze the outcome in patients who have undergone multivisceral resection (MVR) for locally advanced gastrointestinal stromal tumors (GISTs), and identify the risk factors for tumor recurrence and postoperative morbidity. Sixty-four patients who operated for locally advanced GISTs with MVR in PPeking University Cancer Hospital Sarcoma Center (PUCHSC) between 2013 and 2021 were identified. Clinicopathologic characteristics, surgical outcomes, recurrence, and 5-year recurrence-free and overall survival were evaluated. The mean age of the patients was 60 years. Mean tumor size was 11.1 cm. Complete resection was achieved in all patients. The estimated 5-year recurrence-free and overall survival were 86.6% and 90.0%, respectively. Independent factor of recurrence following surgery was mitotic count on multivariate analysis. Overall postoperative morbidity was 53.1% (*n* = 34). Severe morbidity was 21.9% (*n* = 14). The most common severe complication was clinically relevant pancreatic fistula (*n* = 12, 18.8%), followed by anastomotic leakage (*n* = 4, 6.3%) and Intraabdominal abscess (*n* = 4, 6.3%). Multivariate analysis showed that preoperative imatinib therapy could reduce overall morbidity. Long operation time, combined colectomy and pancreatectomy were independent risk factors for postoperative severe morbidity. Compared to partial pancreatectomy, pancreaticoduodenectomy (PD) was significantly increased the incidence of severe morbidity. In conclusion, compared to systemic therapy alone, the outcome of locally advanced GISTs after MVR was more favorable. Despite the high overall morbidity, the postoperative severe morbidity and mortality of MVR were acceptable. Preoperative imatinib therapy should be recommended whenever possible. Combined pancreatectomy and colectomy are associated with significant postoperative severe morbidities. PD should be thoroughly discussed and be subject to MDT approach before surgery.

Keywords gastrointestinal stromal tumors; multivisceral resection; postoperative morbidity; pancreatectomy; surgical outcomes

1. Introduction

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the gastrointestinal tract, and surgery was the mainstay of curative treatment. Although the majority of GISTs could underwent minimal invasive surgery at presentation, a significant number of GISTs are locally advanced, requiring more challenging and complex operations. In a recent study, 11% of 908 GISTs required multivisceral resection (MVR) to achieve complete resection (*1*).

The long-term outcome of unresectable locally advanced GISTs was extremely poor, with a median overall survival time of 3.9 years and 10-year overall survival rates of 20% (*2*). Although imatinib therapy prior to surgery was recommended by most guidelines, which may play an important role by downsizing the tumor, in this way decreasing the extent of resection, unfortunately the reported response rate was not satisfactory (SD/PD cases ranged from 18.6-83%) (*3-5*). Once downsizing failed, MVR remains the only chance of cure. The reported 5-year OS for locally advanced GISTs after MVR ranged from 66.9-80.2% (*6-8*). However, the risk factors associated with recurrence were not well described.

In addition, the safety of MVR remains controversial. In 2015, a retrospective study was reported by Racz (*6*), who compared the perioperative outcomes of 110 patients who required MVR versus single-organ resection (SOR) for GISTs, and concluded that MVR had more

complications than SOR. However, another study with opposite conclusions was published 2 years later. With a series of 187 GISTs, there was no significant difference in in-hospital morbidity and mortality on comparison of MVR versus SOR groups (*7*).

The aim of the study was to analyze the short and long-term outcome of patients who underwent MVR for locally advanced GISTs and identify the risk factors for tumor recurrence and postoperative morbidity.

2. Materials and Methods

2.1. Patients

The study was carried out under the approval of the Ethics Committee of Peking University Cancer Hospital. All patients gave informed consent according to the procedures required by the Institutional Review Board of Peking University Cancer Hospital and Institute and in accordance with the Declaration of Helsinki. All consecutive patients who operated for GISTs with MVR in Peking University Cancer Hospital Sarcoma Center (PUCHSC) between 2013 and 2021 were retrospectively investigated. Clinicopathological data were reviewed from the medical records. All patients had complete preoperative imaging (thorax, abdomen, and pelvic CT scan or MRI) and an image-guided percutaneous coaxial core needle biopsy. Decisions were made by multi-disciplinary team (MDT), and operations were performed by same surgical group. Histopathological results were systematically confirmed by an expert pathologist in soft tissue sarcomas.

2.2. Postoperative morbidities and follow-up

Postoperative morbidities (POM) were graded according to the seven grades of the Clavien-Dindo classification (I, II, IIIa, IIIb, IVa, IVb, and V), and considered severe in case of grade ≥ 3. Postoperative pancreatic fistulas (POPF) were reported according to the International Study Group for Pancreatic Fistula (ISGPF) score, and grade B and C were considered "severe". The patients were prospectively followed with clinical examination, chest X-ray, and abdominopelvic computed tomography (CT) or magnetic resonance imaging (MRI) every three months for the first two years, every six months for the next three years, and yearly thereafter.

2.3. Statistical analysis

Patient characteristics, operative factors, and type of MVR were compared between patients who did and did not experience severe morbidity. Univariate and multivariate logistic regression analyses were conducted to identify independent risk factors for severe morbidity. Overall survival (OS) was defined as the time from surgery to last follow-up or death. Disease-free survival (DFS)was defined as the time from surgery to recurrence or metastasis, last follow-up or death, whichever occurred first. OS and DFS were estimated by Kaplan–Meier method. Statistical analysis was performed using SPSS 22.0.

3. Results

3.1. Patient characteristics

Sixty-four patients with locally advanced GISTs underwent MVR in our institution between 2013 and 2021. Median age at surgery was 60 years. Twenty-six (41%) patients had received neoadjuvant imatinib therapy. Complete resection was achieved in all patients. The mean tumor size was 11.1 cm, and median number of resected contiguous organ for each patient was 2. The most common resected contiguous organ was pancreas (62.5%), followed by spleen (43.8%) and colon (43.8%). Forty-two (66%) patients received adjuvant imatinib therapy within one month after surgery. Demographic and clinicopathological data are summarized in Table 1. The median follow-up was 55 months.

3.2. Postoperative morbidity

Postoperative overall morbidity was 53.1% (*n* = 34), and severe morbidity was 21.9% (*n* = 14). The most common

Table 1. Demographic and clinico-pathological data

Characteristics	*n* = 64 (100%)
Sex: male/female [ratio]	30/34 [0.88]
Median age, years [range]	60 [29–80]
Tumor site	
Gastric	32 (50)
Small bowel	32 (50)
Preoperative imatinib therapy	26 (41)
Postoperative imatinib therapy	42 (66)
Surgery	
Multiviceral resection (MVR)	64 (100)
Median number of resected contiguous organ [range]	2 [1–6]
Associated resections	
Pancreas	40 (62.5)
Spleen	28 (43.8)
Colon	28 (43.8)
Diaphragm	10 (15.6)
Liver	8 (12.5)
Other (lung, kidney, adrenal gland, uterus, bladder)	16 (25)
Median operative time (min) [range]	325 [130–518]
Median perioperative bleeding (mL) [range]	400 [20–2300]
Pathological finding	
Mean tumor size at resection specimen, cm [range]	11.1 [5–26]
Complete resection	64 (100)
Microscopic organ involvement Mutational status	20 (31.3)
KIT exon 11 mutation	40 (62.5)
KIT exon 9 mutation	14 (21.9)
KIT exon 13 mutation	4 (6.3)
PDGFRA exon 18	4 (6.3)
Wild-type	2 (3.1)

severe complication was clinically relevant pancreatic fistula (n = 12, 18.8%), followed by anastomotic leakage (n = 4, 6.3%) and Intraabdominal deep abscess (n = 4, 6.3%). Most severe complications could be managed by percutaneous drainage successfully, and only two patients (1.6%) required surgical reintervention for acute peritonitis secondary to anastomotic leakage and hemorrhage. Postoperative complications are illustrated in Table 2. Multivariate analysis showed that preoperative imatinib therapy was independent risk factor for overall morbidity. Long operation time, combined colectomy and pancreatectomy were independent risk factors for severe morbidity (Table 3). More patients had received neoadjuvant imatinib in non-morbidity group (53.3% vs 29.4%), and patients in severe morbidity group were more likely to have combined pancreatectomy (92.9% vs 54%) and colectomy (78.6% vs 34.0%,) when compared with patients in non-severe morbidity group.

Among patients who had combined pancreatectomy, the majority received distal pancreatectomy (DP) (n = 24, 60%), followed by pancreaticoduodenectomy (PD) (n = 8, 20%), and partial pancreatectomy (PP) (n = 8, 20%). Compared to PP group, severe morbidity in the PD group was significantly higher (p = 0.024), while there was no difference between DP and PP groups (p = 0.468) (Table 4).

Table 2. Postoperative complications

Cases (n)	64 (100%)
Overall morbidity	34 (53.1)
Severe morbidity (Clavien- Dindo III–IV or POPF Grade B and C*)	14 (21.9)
Postoperative Pancreatic Fistula	12 (18.8)
Anastomotic leakage	4 (6.3)
Intraabdominal deep abscess	4 (6.3)
Postoperative Hemorrhage Grade C	2 (3.1)
Acute renal failure	1 (1.6)
Reoperation	2 (3.1)
Postoperative death (day 90)	0

3.3. DFS and OS

The estimated OS and RFS at 5 years were 90.0% and 86.6%, respectively. Eight patients experienced tumor recurrence. Univariate analysis showed that tumor size, mitotic rate and postoperative imatinib therapy was associated with tumor recurrence. However, only high mitotic rate was proved to be independent risk factor by Multivariate analysis (Table 5).

4. Discussion

Locally advanced GISTs represent a clinical challenge, and the prognosis of unresectable cases was extremely poor. In our study cohort, 64 patients underwent MVR for locally advanced GISTs. The 5-year OS and DFS were 90% and 86.6%, which confirmed that MVR could do benefit to selected patients.

In this series, the common indications for MVR include downsizing failure, emergent operations and judgment difficulty. Firstly, some locally advanced GISTs cannot be downsized successfully by neoadjuvant imatinib. The reported response rate of SD/PD cases ranged from 18.6-83% in different studies (*3-5*). For patients of downsizing failure, surgical complexity has not decreased, and MVR is unavoidable. Secondly, GISTs may present with acute abdomen, obstruction, perforation or rupture and peritonitis. In the event of such an emergency, MVR may be forced. According to a

Table 4. Analysis of different type of pancreatectomy for severe morbidity

Items	Severe morbidity	Non-severe morbidity	P-value
No. of patients	13	27	
Partial pancreatectomy	1	7	-
Distal pancreatectomy	6	18	0.468
Pancreaticoduodenectomy	6	2	0.024

Table 3. Univariate and multivariate analysis of risk factors for overall and severe morbidity

Items	Overall Morbidity group	Non-morbidity group	Univariate analyses P-value	Multivariate analyses P-value	Severe morbidity group	Non-severe morbidity group	Univariate analyses P-value	Multivariate analyses P-value
No. of patients	34	30			14	50		
Age (years)	57.7	57.3	0.933	-	56.7	57.7	0.811	-
Sex (male)	12 (35.2%)	18 (60.0%)	0.143	-	6 (42.9%)	24 (48.0%)	0.529	-
Preoperative Imatinib	10 (29.4%)	16 (53.3 %)	0.003	0.048	7 (50%)	19 (38.0%)	0.347	-
Tumor site			0.316	-			0.546	-
Stomach	16 (47.1%)	16 (53.3%)			6 (42.9 %)	26 (52.0%)		
Small intestine	18 (52.9%)	14 (46.7 %)			8 (57.1%)	24 (48.0%)		
Mean tumor size (cm)	9.9	12.5	0.008	0.065	8.6	11.8	0.054	-
Operative factors								
Resected organ ≥ 3	6 (17.6%)	10 (33.3%)	0.285	-	4 (28.6%)	12 (24.0%)	0.727	-
+Pancreatectomy	30 (88.2%)	10 (33.3%)	0.247	-	13 (92.9%)	27 (54.0%)	0.025	0.003
+splenectomy	16 (47.1%)	12 (40.0%)	0.950	-	6 (42.9%)	22 (44%)	0.939	
+colectomy	16 (47.1%)	12 (40.0%)	0.617	-	11 (78.6%)	17 (34.0%)	0.048	0.016
Operation time (min)	350	296	0.381	-	403	302	0.001	0.014
Blood loss (mL)	819	467	0.976	-	1000	557	0.045	0.272

Table 5. Univariate and multivariate analysis of RFS

Items	Non-recurrence group	Recurrence group	Univariate analyses *P*-value	Multivariate analyses *P*-value
No. of patients	56	8		
Age (yrs)	56.8	63.0	0.180	-
Sex (male)	29 (51.2%)	1 (12.5%)	0.067	0.082
Preoperative Imatinib	22 (39.3%)	4 (50%)	0.850	
Emergency	4 (7.1%)	2 (25%)	0.130	0.099
Tumor site			1.000	-
Stomach	28 (50%)	4 (50%)		
Small bowel	28 (50%)	4 (50%)		
Tumor size (cm)			0.026	0.418
< 15	48 (85.7%)	4 (50%)		
≥ 15	8 (14.3%)	4 (50%)		
Mitotic rate per 50 HPF			0.006	0.044
> 5	12 (21.4%)	6 (75%)		
≤ 5	44 (78.6%)	2 (25%)		
Microscopic organ involvement	18 (32.1%)	2 (25%)	0.685	
Postoperative Imatinib	40 (71.4%)	2 (25%)	0.020	0.206
Overall morbidity	31 (55.4%)	3 (37.5%)	0.351	-
Severe morbidity	11 (19.6%)	3 (37.5%)	0.264	-
Operative factors				
Resected organ ≥ 3	14 (25%)	2 (25%)	1.000	-
Operation time (min)	325	326.5	0.966	-
Blood loss (mL)	663	450	0.405	-

report by Magdy, among 92 GISTs-related emergencies, 2 patients required MVR (*9*). Finally, in some cases, although downsized successfully, the tumor is still close to neighboring organs. It is difficult to make a sound judgment whether the tumor is adherence or infiltration of the surrounding organs. To ensure complete resection and avoid iatrogenic tumor rupture, en bloc resection of adjacent organs is still required (*10*). Other less common causes of MVR in this study include being misdiagnosed as primary retroperitoneal sarcoma, intolerance to imatinib and wide-type GISTs. In addition, microscopic contiguous organ involvement could be observed in more than 30% cases, proving that MVR is sometimes necessary to achieve complete resection.

Established prognostic factors for GISTs are tumor site, tumor size, mitotic count and tumor rupture (*11-13*). However, on multivariate analysis of this study, only high mitotic count was associated with recurrence. More tumors originated in the duodenum and the larger tumor volumes was observed in this series, which might be one of the reasons for the discrepancy. DeMatteo and colleagues proved that size > 10 cm was independently prognostic factors of RFS for GISTs (*13*). However, most tumors in our study were larger than 10cm. therefore, the analysis value we chose was 15 cm. On univariate analysis, larger tumor size was associated with tumor recurrence, but not proved by multivariate analysis.

Tumor rupture was considered to be associated with a substantially higher risk of tumor recurrence. Patients with intraoperative rupture of GIST into the peritoneal cavity had a risk of recurrence (*14*). In the present study, there was no tumor rupture occurred and the 5-year RFS in patients who underwent MVR for locally advanced GISTs was excellent, which might be due to the aggressiveness of MVR. Firstly, we do not attempt to separate the tumor from the surrounding involvement organs in order to avoid exposing the tumor and make a resection similar to "compartment resection". Secondly, an "anterior approach" was routinely used to avoid compression and rotate the tumor. Undoubtedly, such an "no-touch" surgical technique could minimize the risk of iatrogenic tumor rupture.

In this study, MVR was proved to be a more complicated surgical procedure The median number of resected contiguous organ for each patient was 2, and half of the tumors originated in the duodenum and the mean tumor size was 11.1 cm, leading a result in much higher proportion of combined pancreatectomy. The morbidity of pancreatectomy remains high even in high-volume centers. In a report from the Transatlantic RPS Working Group, the overall morbidity was 64% for patients with soft tissue sarcoma who underwent surgery requiring pancreas resection (*15*). Therefore, the overall morbidity in this cohort was higher than reports in the literature (*16-18*). The benefits of MVR must be balanced against the risks associated with the operation.

Fortunately, preoperative imatinib could significantly reduce the overall morbidity. Most GISTs are fragile and hypervascular, leading the high risk of bleeding and tumor rupture. Imatinib can reduce the blood supply and make the tumor robust, which may facilitate surgical procedure and reduce postoperative complications (*17*). In a retrospective study of 25 advanced GISTs patients who underwent surgery after preoperative imatinib therapy, no postoperative complications appeared (*18*). In a prospective phase II study, designed to evaluate safety and efficacy of neoadjuvant imatinib for patients with advanced primary and metastatic/recurrent operable

GISTs, the complications of surgery were also minimal (*4*). Based on these results, preoperative imatinib therapy should be recommended whenever possible.

Our data showed that pancreatectomy was associated with significant severe morbidity of MVR. Twelve (18.8%) patients had a clinically relevant (grade B or C) POPF, which is slightly higher than data of ISGPF (*19*). It might be related to normal pancreatic duct, soft pancreas tissue and imatinib associated edema for GISTs. Two patients had a postoperative hemorrhage (grade C, 3.1%) which was comparable to the results in the literature (*20*) and both of them had undergone a combined PD. Compared to partial pancreatectomy, PD was more complicated and required more operation time, which was another independent risk factor for severe morbidity after MVR in this study. Therefore, in our center PD should be thoroughly discussed and the patient should be fully informed about the risks before surgery.

Furthermore, nearly 40% of patients had combined colectomies in the cohort. Addition of colectomy to pancreatectomy could aggravate the severity of pancreatic fistula. Swchartz (*21*) reported that the 90-day morbidity and mortality rate in patients of simultaneous pancreatectomy with colectomy were 61% and 14% respectively, which was much higher than pancreatectomy alone (42% and 3%, $P < 0.01$). To mitigate mortality risk, a diverting loop ileostomy was selectively done for patients of MVR requiring combined colectomy. By reducing the enteral stream across the anastomosis, the morbidity associated with a colonic leak could potentially be avoided. Although the patients had to undergo stomal closure three months later, no fistula-related perioperative deaths occurred in this series.

In conclusion, compared to systemic therapy alone, the outcome of locally advanced GISTs after MVR was more favorable. Despite the high overall morbidity, the postoperative severe morbidity and mortality of MVR were acceptable. Preoperative imatinib therapy should be recommended whenever possible. Combined pancreatectomy and colectomy are associated with significant postoperative severe morbidities. PD should be thoroughly discussed and be subject to MDT approach before surgery.

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[§]These authors contributed equally to this work.

*Address correspondence to:
Chunyi Hao, Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education, Beijing), Department of Hepato-Pancreato-Biliary Surgery, Peking University Cancer Hospital and Institute, #52 Fucheng Road, Haidian District, Beijing 100142, China.
E-mail: haochunyi@bjmu.edu.cn

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Brief Report

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Emotional journey of patients with specified intractable diseases in Japan

Hiroyuki Tanaka[1,2,*], Mikiko Shimaoka[1]

[1] Graduate School of Health Innovation, Kanagawa University of Human Services, Kawasaki City, Japan;
[2] CMIC Inizio Co., Ltd., Tokyo, Japan.

SUMMARY This study aimed to depict the emotional journey of Japanese patients with specific intractable diseases facing challenges associated with a delayed diagnosis. Specifically, our focus was on elucidating the emotional journey of patients and identifying the unmet needs caused by a delayed diagnosis. We conducted a web-based survey targeting 179 patients with 11 specified intractable diseases. They reported their emotional states during each journey phase using a 10-point scale. The results revealed that the period from noticing bodily changes to clinic visits was characterized by the most negative emotional states. Furthermore, the patients experienced a gradual shift towards positive emotional states as they decided to complete a consultation at a specialized hospital. They reached their most positive emotional states when they received a definitive diagnosis, subsequent treatment, and care. The thematic classification of emotional changes at the time of definitive diagnosis showed that "relief" was the most prevalent emotion (41.9%), followed by "no change" (19.9%), "anxiety" (14.0%), "shock" (13.4%), and "resignation" (6.5%). Additionally, when classifying the thematic changes in emotions during the period of bodily changes and clinic visits, "frustration" was the most common (51.3%), followed by "fear and anxiety" (43.6%). Patients tended to be most psychologically distressed during the period leading up to the definitive diagnosis. These results reveal that patients with intractable diseases are seeking a fast and accurate diagnosis, and that achieving these is a key unmet need for the patients.

Keywords patient journey, emotional journey, definitive diagnosis, diagnostic delay

1. Introduction

Although rare diseases individually affect a small number of patients, they collectively comprise over 7,000 conditions worldwide, affecting an estimated 350 million individuals. Patients with rare diseases face the challenges of difficult diagnoses and limited access to specialized medical expertise; together, these issues result in misdiagnoses or delayed diagnoses for many patients (*1,2*). Consequently, patients with rare diseases often face significant difficulties in their daily lives, highlighting the need to address this public health challenge (*3*).

In Japan, rare diseases are generally defined as medical conditions involving less than 50,000 patients. Rare diseases that do not meet this Ministry of Health, Labour and Welfare criterion and that have established objective diagnostic criteria are designated as "specified intractable diseases". The government recognizes these rare diseases and includes them in its medical expense subsidy programs (*4*). Despite these efforts to establish a medical support network for rare diseases and reduce the amount of time for definitive diagnosis in Japan, many patients still wait a long time before receiving a confirmed diagnosis (*5*). This matters because early diagnosis is crucial for patients with rare diseases. Even in cases in which effective treatments are lacking, early diagnosis enables the implementation of strategies to manage or slow disease progression, contributing to potential improvements in a patient's quality of life (QOL) (*6,7*).

Therefore, this study aimed to capture the emotional states of patients throughout the entire medical process – from the onset of symptoms through to diagnosis, treatment, and care. To capture the experiences and emotional changes of patients at each stage, we employed the widely used method of depicting the "patient journey". Several studies targeting patients with rare diseases have used this approach (*8,9*). However, most of these studies illustrated the experiences of a few representative patients for each disease based on non-structured interviews. Although this approach is effective for exploring diverse patient journeys and gaining deep

insights into patient experiences, it can only survey a limited number of patients. Furthermore, interviewing individual patients may also reveal strong personal emotions related to each patient's specific situation, which can make it challenging to capture the overall emotional landscape of patients with a particular disease. Few studies on rare diseases have targeted a large number of patients to capture their emotional changes and identify their unmet needs. Therefore, this study aimed not to focus on a very limited number of patients through interviews but rather to comprehensively capture emotional changes from a multitude of patients using a web-based survey method, with the goal of identifying the unmet needs of patients. We selected rare diseases based on the results of our previous research, which identified rare diseases associated with a high likelihood that patients would delay seeking medical care despite feeling abnormal symptoms and wait a long time before receiving a definitive diagnosis (*10*).

2. Materials and Methods

2.1. Study population

As noted above, our previous research in Japan targeting patients with specific intractable diseases revealed that they tended to wait a long time after the onset of abnormal symptoms to seek medical care; further, we also found that these patients tended to experience long delays (*e.g.*, over a year) in receiving a definitive diagnoses (*10*). Building on these findings, this study focused on diseases in which these observed outcomes occurred frequently.

We conducted a web-based questionnaire in November 2023 using patient panels owned by Rakuten Insight, Inc. Eleven designated intractable diseases (Crohn's disease, Sjögren's syndrome, polycystic kidney disease, IgA nephropathy, systemic lupus erythematosus, Parkinson's disease, idiopathic dilated cardiomyopathy, multiple sclerosis/neuromyelitis optica, spinocerebellar degeneration (excluding multiple system atrophy), idiopathic interstitial pneumonia, and eosinophilic sinusitis) were targeted, and participants were recruited from patient panels maintained by the company. The survey methodology involved an Internet-based questionnaire. The initial sample comprised 212 respondents. After excluding cases deemed analytically unfeasible, the final sample for the analysis included 179 respondents (this was a valid final sample size).

2.2. Questionnaires

In this study, a web-based questionnaire approach was utilized instead of interviews to survey participants. This approach allows for broad participation by reaching out to a wide range of participants. Additionally, the use of a questionnaire ensures that uniformity is maintained in the wording of the questions and the scale, providing the advantage of achieving consistency in patient responses. The participants were informed about the purpose of the research, and their participation implied consent. The questionnaire asked them to state their demographic information, such as the name of the disease with which they had been diagnosed and their age, sex, marital status, and educational background. The participants selected their responses from predefined options.

The survey focused on emotional states at various stages of the medical journey, including the onset of awareness of bodily changes, emotions felt upon learning about bodily changes from health check-up results, emotions experienced from the onset of feeling bodily changes to visiting a clinic, emotional states experienced during the transition from a clinic to a specialized hospital, emotions felt during the initiation of examinations at a specialized hospital, emotional states at the time of definitive diagnosis at a specialized hospital, and emotions experienced at the commencement of treatment and care. Respondents were asked to rate these emotional states on a scale of 1–10, with 5 representing a neutral or usual emotional state. Additionally, the participants were encouraged to provide free-text descriptions of their emotions at each stage and elaborate on any changes in their emotions upon receiving a confirmed diagnosis (Supplemental Table S1, *http://www.irdrjournal.com/action/getSupplementalData.php?ID=181*).

2.3. Analysis

For the obtained 10-point emotional states, the average value was calculated and utilized as the "emotional status". Given the subjective nature of individual responses at each stage, we chose not to account for variability in the numerical values when calculating the average.

Qualitative data collected from the web survey were subjected to thematic analysis (*11*) using MAXQDA 2022, software by VERBI GmbH. This approach involves a thorough examination of the dataset to deconstruct qualitative data, reveal patterns of underlying meaning, and thereby identify comprehensive themes. Codes were assigned to themes, which were further clustered. Themes related to emotional changes upon receiving a confirmed diagnosis were categorized into "relief", "anxiety", "shock", "no change", and "other factors". Themes associated with the stage where participants reported the most negative emotions before visiting a specialized hospital were classified into "frustration", "fear and anxiety", "aversion to visiting the hospital", and "other factors". The quoted passages in the emotional journey were included to facilitate the understanding of the phenomenon.

2.4. Ethical approval

This study, conducted using a fully anonymized questionnaire survey, underwent an ethical review and was approved by the Research Ethics Review Committee of the School of Health Innovation at Kanagawa University of Human Services. The application was submitted according to prescribed procedures before the commencement of the study, and ethical approval was granted (registration number: SHI No. 35). In addition, informed consent was obtained from all of participants on the website.

3. Results and Discussion

Table 1 presents the characteristics of the participants, with the number of patients in each disease group ranging from 9 to 25. While the overall sex distribution was 62.6% male, diseases such as Sjögren's syndrome, systemic lupus erythematosus, and multiple sclerosis/optic neuromyelitis spectrum disorder were more prevalent among females.

Next, we depicted the emotional journey of patients from the onset of recognizing bodily anomalies to receiving treatment and care (Figure 1). The emotional status was 2.50 at the stage of recognizing bodily anomalies, 2.45 when visiting clinics, and 2.95 upon arrival at the specialized hospitals. Thus, the patients' emotional states during the period from recognizing bodily anomalies to visiting specialized hospitals were predominantly negative. Moreover, their emotional status gradually increased as they entered the diagnostic phase (3.23), reached a confirmed diagnosis (3.24), and began treatment and care (3.84). In sum, these results suggest that patients experience a positive shift in their emotional status upon entering the diagnostic phase at a specialized hospital, which intensifies during the definitive diagnosis and treatment phases. Notably, patients who visited specialized hospitals directly after receiving their health check results maintained an emotional status between 4.14 and 4.35, with minimal fluctuations.

Furthermore, we classified the participants' emotional responses to a definitive diagnosis into six themes: relief, anxiety, shock, no change, and other factors. The results in Figure 2 show that relief was the most prevalent theme (41.9%), followed by no change (19.9%), anxiety (14.0%), shock (13.4%), and resignation (6.5%). After a definitive diagnosis, participants experienced a mix of shock and relief, with many expressing relief at the end of their understanding of the cause of their symptoms. Interestingly, 19.9% of the participants reported no significant emotional change, positively accepted their diagnosis, and approached treatment and care from a forward-looking perspective.

Moreover, we explored specific emotions during the period when patients felt most negatively about their journeys, namely, leading up to specialized hospital visits. Responses were categorized into four themes: frustration, fear, anxiety, aversion to hospital visits, and other factors (Figure 3). Frustration was the predominant theme (51.3%), followed by fear and anxiety (43.6%). The results indicated that patients experiencing frustration were often unable to identify the cause of their symptoms even after multiple clinic visits and tests, which contributed to their negative emotional state. Additionally, the fear of deteriorating health during this process proved to be a significant contributor to negative emotions. One of the causes for the decline in the emotional state of these patients is the delay in referrals from general practitioners (GPs), who often have the opportunity to initially diagnose patients with rare diseases, to specialized hospitals. Many comments from patients indicated that GPs did not refer them to specialized hospitals without suspecting a rare disease. It became evident that addressing how to promptly refer patients from GPs to specialized hospitals is a crucial issue that needs to be resolved. These findings provide crucial insights into the unmet needs of patients with rare diseases; notably, they emphasize the importance of an early confirmed diagnosis for QOL.

In our previous study, we found that a prolonged duration before a definitive diagnosis leads to a decline in physicians' trust (*12*). A weak trust relationship may result in patients being less inclined to communicate detailed symptoms to physicians, potentially hindering a thorough understanding of their conditions. In the case of rare diseases, expediting the steps to visiting a specialized hospital before reaching a definitive diagnosis is crucial. GPs play a significant role in this work; however, GPs should build strong relationships with specialized hospitals to ensure they can efficiently diagnose rare diseases while treating multiple patients.

Further, it is essential for patients to contemplate what health information they should communicate to physicians. Notably, social media initiatives have recently emerged that enable patients with similar health issues to actively exchange opinions and find clinicians with knowledge of rare diseases (*13*). Along these lines, Yamaguchi *et al.* (*14*) explored whether social media postings of the medical history of rare disease patients may shorten the timeline for diagnosis and treatment. Such endeavors not only benefit researchers but may also motivate undiagnosed patients worldwide to seek medical attention.

This study has two strengths. First, it demonstrates the ability to depict the journey of a patient with a rare disease and capture changes in their emotional status at each stage; this approach can notably be used to identify the unmet needs of patients with rare diseases. Second, the study evidences that a web survey, which allowed for an analysis with a large sample size, is a valuable approach for capturing changes in patients' emotional statuses.

However, this study has several limitations. If it is

Table 1. Characteristics of the study sample

Characteristic	Study sample		Crohn's disease		Idiopathic interstitial pneumonia		Eosinophilic sinusitis		Sjögren's syndrome		Polycystic kidney disease		IgA nephropathy		Systemic lupus erythematosus		Parkinson's disease		Idiopathic dilated cardiomyopathy		Multiple sclerosis/ neuromyelitis optica		Spinocerebellar degeneration	
	n	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)
Total	179	(100.0)	22	(100.0)	9	(100.0)	13	(100.0)	16	(100.0)	20	(100.0)	24	(100.0)	25	(100.0)	14	(100.0)	14	(100.0)	13	(100.0)	9	(100.0)
Sex																								
Male	112	(62.6)	18	(81.8)	9	(100.0)	12	(92.3)	2	(12.5)	15	(75.0)	15	(62.5)	6	(24.0)	9	(64.3)	13	(92.9)	6	(46.2)	7	(77.8)
Female	67	(37.4)	4	(18.2)	0	(0.0)	1	(7.7)	14	(87.5)	5	(25.0)	9	(37.5)	19	(76.0)	5	(35.7)	1	(7.1)	7	(53.8)	2	(22.2)
Age																								
< 29 years	3	(1.7)	0	(0.0)	0	(0.0)	0	(0.0)	2	(12.5)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	1	(7.7)	0	(0.0)
29–49 years	40	(22.3)	10	(45.5)	0	(0.0)	2	(15.4)	4	(25.0)	5	(25.0)	5	(20.8)	6	(24.0)	1	(7.1)	2	(14.3)	3	(23.1)	2	(22.2)
50–59 years	66	(36.9)	9	(40.9)	1	(11.1)	3	(23.1)	6	(37.5)	8	(40.0)	10	(41.7)	12	(48.0)	2	(14.3)	3	(21.4)	7	(53.8)	5	(55.6)
60–69 years	50	(27.9)	2	(9.1)	5	(55.6)	8	(61.5)	2	(12.5)	2	(10.0)	9	(37.5)	4	(16.0)	7	(50.0)	7	(50.0)	2	(15.4)	2	(22.2)
> 69 years	20	(11.2)	1	(4.5)	3	(33.3)	0	(0.0)	2	(12.5)	5	(25.0)	0	(0.0)	3	(12.0)	4	(28.6)	2	(14.3)	0	(0.0)	0	(0.0)
Marriage																								
Married	118	(65.9)	12	(54.5)	8	(88.9)	9	(69.2)	7	(43.8)	15	(75.0)	20	(83.3)	15	(60.0)	9	(64.3)	10	(71.4)	8	(61.5)	5	(55.6)
Other	61	(34.1)	10	(45.5)	1	(11.1)	4	(30.8)	9	(56.3)	5	(25.0)	4	(16.7)	10	(40.0)	5	(35.7)	4	(28.6)	5	(38.5)	4	(44.4)
Education Background																								
Junior school	4	(2.2)	0	(0.0)	0	(0.0)	1	(7.7)	0	(0.0)	1	(5.0)	1	(4.2)	0	(0.0)	1	(7.1)	0	(0.0)	0	(0.0)	0	(0.0)
High school	46	(25.7)	5	(22.7)	0	(0.0)	6	(46.2)	5	(31.3)	4	(20.0)	4	(16.7)	10	(40.0)	2	(14.3)	6	(42.9)	4	(30.8)	0	(0.0)
Professional school	28	(15.6)	4	(18.2)	1	(11.1)	1	(7.7)	0	(0.0)	4	(20.0)	3	(12.5)	4	(16.0)	2	(14.3)	1	(7.1)	5	(38.5)	3	(33.3)
Junior college	20	(11.2)	3	(13.6)	0	(0.0)	0	(0.0)	3	(18.8)	1	(5.0)	4	(16.7)	3	(12.0)	1	(7.1)	2	(14.3)	2	(15.4)	1	(11.1)
Bachelor's degree	68	(38.0)	7	(31.8)	7	(77.8)	5	(38.5)	6	(37.5)	9	(45.0)	7	(29.2)	8	(32.0)	8	(57.1)	4	(28.6)	2	(15.4)	5	(55.6)
Master's degree and above	12	(6.7)	3	(13.6)	1	(11.1)	0	(0.0)	1	(6.3)	1	(5.0)	5	(20.8)	0	(0.0)	0	(0.0)	1	(7.1)	0	(0.0)	0	(0.0)
Other	1	(0.6)	0	(0.0)	0	(0.0)	0	(0.0)	1	(6.3)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
Medical visit pattern																								
Initially visited the clinic	115	(64.2)	17	(77.3)	3	(33.3)	10	(76.9)	11	(68.8)	6	(30.0)	15	(62.5)	18	(72.0)	11	(78.6)	13	(92.9)	7	(53.8)	4	(44.4)
Visited specialized hospitals from the beginning	43	(24.0)	3	(13.6)	3	(33.3)	2	(15.4)	3	(18.8)	9	(45.0)	8	(33.3)	4	(16.0)	2	(14.3)	1	(7.1)	4	(30.8)	4	(44.4)
Others	21	(11.7)	2	(9.1)	3	(33.3)	1	(7.7)	2	(12.5)	5	(25.0)	1	(4.2)	3	(12.0)	1	(7.1)	0	(0.0)	2	(15.4)	1	(11.1)

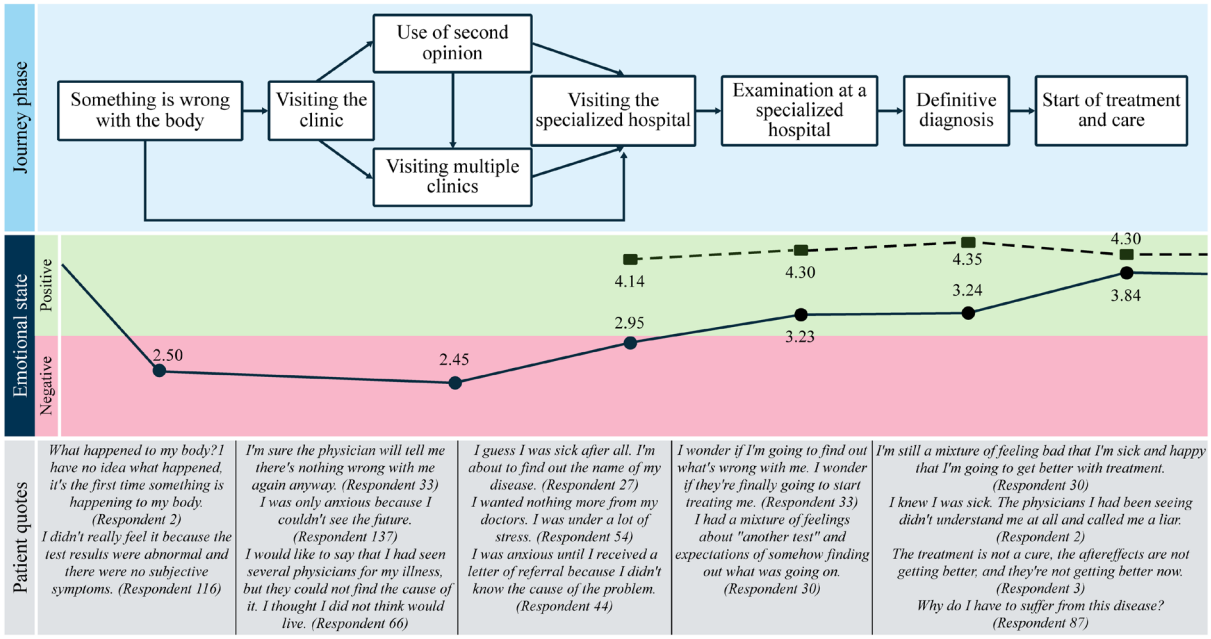

Figure 1. Emotional journey of patient with intractable disease.

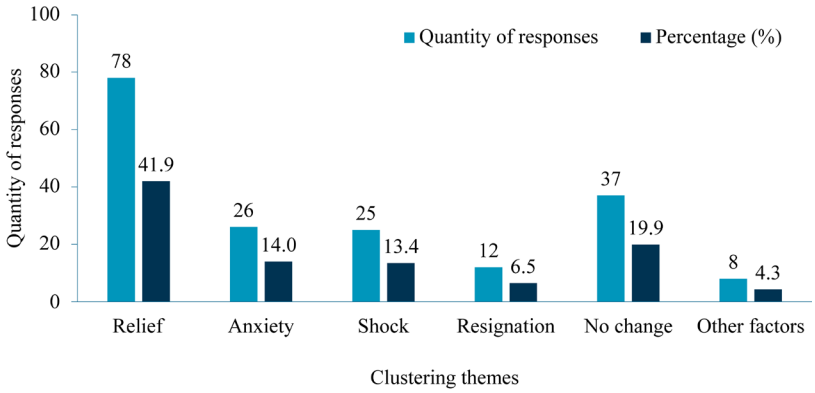

Figure 2. The emotional shift at the time of the definitive diagnosis.

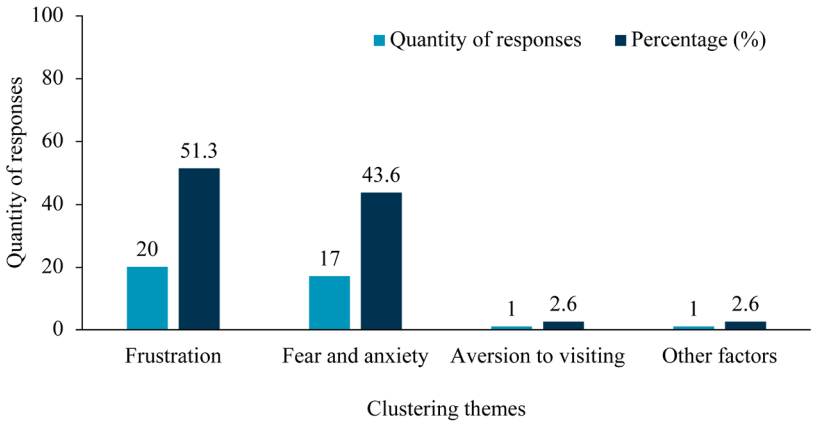

Figure 3. Clustering themes of specific emotions during the period when patients felt most negatively until they visited a specialized hospital.

anticipated that there are numerous patient journeys for each persona, thorough pre-investigation is necessary. The method of understanding the emotional states of patients through web surveys, as done in this study, may be more suitable for diseases that follow a somewhat typical medical course. In other words, this study focused on the challenges in the definitive diagnosis of specific rare diseases in Japan, and the selected disease group

allowed for some degree of prior prediction of the patient journey. In future research, it is necessary to verify the effectiveness of this survey method using a broader range of disease groups and sample sizes.

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**Address correspondence to:*
Hiroyuki Tanaka, Graduate School of Health Innovation, Kanagawa University of Human Services, Research Gate Building TONOMACHI 2-A, 3-25-10 Tonomachi, Kawasaki-ku, Kawasaki City, Kanagawa Prefecture 210-0821, Japan.
E-mail: hiroyuki.public.health@gmail.com

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Brief Report

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Clinical features of extrahepatic portal vein obstruction: Myeloproliferative neoplasms eliminate hypersplenic hematologic changes in extrahepatic portal vein obstruction

Tetsuya Shimizu*, Hiroshi Yoshida, Nobuhiko Taniai, Masato Yoshioka, Yoichi Kawano, Akira Matsushita, Junji Ueda, Takuma Iwai, Takahiro Murokawa, Takashi Ono, Akira Hamaguchi

Department of Gastrointestinal and Hepato-Biliary-Pancreatic Surgery, Nippon Medical School, Tokyo, Japan.

SUMMARY Extrahepatic portal vein obstruction (EHPVO) is a rare disease. Most EHPVO patients are usually referred to a gastroenterologist for intestinal bleeding and hypersplenic thrombocytopenia; however, hypercoagulative diseases may be occult in these patients and require anticoagulation. The purpose of this study was to elucidate the clinical characteristics of EHPVO. We conducted a retrospective analysis of the hospital database, evaluating the medical records of 15 patients (7 males, 8 females, mean age of onset 42.0 years, range 5–74 years). Thirteen of 15 EHPVO patients (86.7%) had intestinal varices. These included 10 esophageal (66.7%), 12 gastric (80.0%), and 6 ectopic varices (40.0%). Nine (60.0%) of 15 had a history of intestinal bleeding. Regarding comorbidities, 5 of 15 (33.3%) suffered from vascular diseases, including acute myocardial infarction, cerebral infarction, pulmonary embolism, Budd–Chiari syndrome, and mesenteric vein thrombosis. The former 3 vascular commodities manifested at less than 32 years of age. Four patients (26.7%) with JAK2V617F mutation were diagnosed as myeloproliferative neoplasm (MPN). 72.3% of EHPVO patients without MPN experienced thrombocytopenic state. No EHPVO patients with MPN experienced thrombo-leukocytopenia. The elevation of white blood cell and platelet counts, and decrease of protein S were seen in EHPVO with MPN, compared with EHPVO without MPN. EHPVO is frequently associated with underlying hypercoagulative factors, causing a dilemma between thrombotic complications and portal hypertensive bleeding. Most EHPVO patients experience an evident thrombocytopenic state due to severe hypersplenism; however, hypersplenic hematologic changes are eliminated in EHPVO with MPN. MPN should be suspected in EHPVO patients negative for thrombo-leukocytopenia.

Keywords extrahepatic portal vein obstruction, myeloproliferative neoplasm, JAK2V617F mutation

1. Introduction

Extrahepatic portal vein obstruction (EHPVO) is an important cause of non-cirrhotic portal hypertension and a rare disorder with an estimated incidence of 6.1 per 1,000,000 inhabitants in Japan (*1*). In Europe, the overall gender-specific incidence rates of EHPVO were reportedly 3.78 and 1.73 per 100,000 inhabitants, in males and females, respectively (*2*). EHPVO is the thrombotic obliteration of the extrahepatic portal vein, with or without involvement of the intrahepatic portal veins or other segments of the splanchnic venous axis. EHPVO is characterized by features of portal hypertension with portal cavernous transformation as a sequel of portal vein obstruction to compensate for the interrupted portal blood flow (*2,3*). The etiology of EHPVO is diverse: however, an association with underlying risk factors for hypercoagulation has been reported.

The majority of patients with EHPVO are usually referred to a gastroenterologist for intestinal bleeding and hypersplenic thrombocytopenia. Gastroenterologists are often faced with a dilemma between fetal intestinal hemorrhage and thrombotic complications, and forced to simultaneously provide hemostatic control for portal hypertensive bleeding and antithrombotic agent administration. This creates a series of medical care issues for every EVPVO patient, including diagnosis, diverse comorbidities, complications, and treatment. The rarity of EHPVO makes controlled studies

impractical, diagnosis difficult, and therapeutic strategies unstandardized.

This retrospective single-center study was conducted to clarify the clinical features of EHPVO and thrombotic comorbidities including hematological and vascular disease to improve diagnostic accuracy and therapeutic efficacy.

2. Patients and Methods

Between January 2000 and July 2023, 15 patients with a diagnosis of EHPVO treated in our hospital were enrolled in this retrospective study. Medical records for all 15 patients were identified and reviewed retrospectively. In all patients, the diagnosis was confirmed by imaging modalities, including ultrasonography, contrast-enhanced computed tomography, angiography, or contrast-enhanced magnetic resonance imaging. Patients with hepatocellular carcinoma, other malignancies, liver cirrhosis or operative history including pancreaticoduodenectomy and choledochotomy were excluded.

The medical records for patient age, gender, body mass index, habit (smoking, and alcohol intake), family history, symptoms at initial onset, comorbidity, laboratory workup, esophagogastroduodenoscopy findings were extracted from patient charts.

Regular blood tests, hepatic and renal function tests were performed in all patients. Thrombotic risk factors of EHPVO, including protein S, protein C, antithrombin III, D-dimer, JAK2V617F mutation, and anti-cardiolipin IgG antibodies, were tested, if needed, by the receiving doctor.

Esophagogastric varices were stratified according to the grading system proposed by the Japan Society for Portal Hypertension (*4*). Gastric varices in gastric body and antrum were included in ectopic varices. The system did not mention grading for ectopic varices; however, ectopic varices (duodenal varices and gastric body varices) were classified according to the same grading system for gastric varices (*4*) in this study.

All study participants provided informed consent and the study was carried out in accordance with the ethical standards set by the Declaration of Helsinki. This study was approved by the hospital ethics committee (B-2022-615).

The data were analyzed using IBM SPSS Statistics® Ver. 28.0.1. Discrete variables were compared using Fisher's exact test. Continuous variables were compared using the Mann-Whitney *U*-test since the sample sizes were relatively small. A p-value < 0.05 was considered significant.

3. Results and Discussion

Fifteen EHPVO patients were included in the analysis. The demographic data and clinical presentation of the EHPVO patients are presented in Table 1. Seven males (46.6%) and 8 females (53.3%); the age distribution of onset ranged from 5 years to 74 years, with a mean age of onset of 42.0 years. Body mass index (kg/m^2) was 23.4 ± 4.2 with a range from 17.6 to 34.2. Of the 15 patients, 6 (40.0%) were smokers, and 7 (46.6%) reported usual alcohol intake.

Initial complaint included 5 hematemesis (33.3%), 5 abdominal pain (33.3%), 2 asymptomatic (13.3%), 1 bloody stool (6.7%), 1 fullness in the left upper quadrant (6.7%), and 1 irregular genital bleeding (6.7%). Regarding disease-associated complications, 13 (86.7%) of 15 EHPVO patients had intestinal varices, including 10 esophageal varices (66.7%), 12 gastric varices in fundus and cardia (80.0%), and 6 ectopic varices (40.0%) with 5 duodenum and 1 gastric body. Nine (60.0%) and 11 (73.3%) of 15 had intestinal bleeding history and variceal red color sign indicating high risk for variceal rupture, respectively. Thrombocytopenia (< 20.0 × 10^4/uL), portal hypertensive gastropathy, ascites, and portal biliopathy was noted in 8 (53.3%), 4 (26.7%), 2 (13.3%), and 2 (13.3%) of 15 EHPVO patients, respectively.

Regarding comorbidities, 5 of 15 (33.3%) suffered from vascular diseases, including 1 acute myocardial infarction (6.6%), 1 cerebral infarction (6.6%), 1 pulmonary embolism (6.6%), 1 Budd-Chiari syndrome (6.6%), and 1 mesenteric vein thrombosis (6.6%). The 3 former vascular commodities manifested at less than 32 years of age. None of the 15 EHPVO patients had associated family history of thrombotic diseases. Four (26.7%) and 3 (20.0%) of 15 patients had hematological diseases of myeloproliferative neoplasm (MPN) and diabetes mellitus, respectively.

Laboratory workup for coagulation inhibitor and causative factors with EHPVO patients are shown in Table 2. Antithrombin III and protein S were deficient in 30.8% (4 of 13 tested patients) and 41.7% (5 of 12 tested patients), respectively. However, no deficiency of protein C was noted in any of the tested EHPVO patients (0.0%, 0 of 12 tested patients). Elevation of D-dimer was found in 13 of 14 tested EHPVO patients (92.9%).

Anti-cardiolipin IgG antibody for screening of antiphospholipid syndrome was negative in all 11 tested patients (0.0%). JAK2V617F mutation, indicating association with MPN, was detected in 36.3% (4 of 11 tested EHPVO patients). All 4 MPN patients diagnosed by a hematologist during EHPVO screening had JAK2V617F mutation. With further hematological scrutiny, 4 MPN patients with EHPVO were diagnosed, respectively, as 2 polycythemia vera (PV), 1 essential thrombocythemia (ET), and 1 myeloproliferative neoplasm, unclassifiable (MPN-U).

Distribution of platelet and white blood cell counts in EHPVO is noted in Figure 1. Eight of 15 EHPVO patients (53.3%) exhibited thrombocytopenia (< 20.0 × 10^4/uL). Eight of 11 EHPVO patients without MPN (72.3%) exhibited a thrombocytopenic state; however,

Table 1. Demographic data and clinical presentation for EHPVO patients

Characteristic	All patients (n = 15)		
Gender			
Male, n (%)	7	(46.6%)	
Female, n (%)	8	(53.3%)	
Age of onset			
mean ± SD (range)	42.0 ± 18.1	(5–74)	
Body mass index (Kg/m^2)			
mean ± SD (range)	23.4 ± 4.2	(17.6–34.2)	
Initial complaint			
hematemesis	5	(33.3%)	
abdominal pain	5	(33.3%)	
none	2	(13.3%)	
bloody stool	1	(6.7%)	
fullness in the left upper quadrant	1	(6.7%)	
irregular genital bleeding	1	(6.7%)	
Disease-associated complications			
upper gastrointestinal varices	13	(86.7%)	
esophageal varices	10	(66.7%)	
gastric varices	12	(80.0%)	
ectopic varices	6	(40.0%)	5 duodenum, 1 gastric body
history of intestinal bleeding	9	(60.0%)	
thrombocytopenia (< 2.0 × 10^4/uL)	8	(53.3%)	
portal hypertensive gastropathy	4	(26.7%)	
ascites	2	(13.3%)	
portal biliopathy	2	(13.3%)	
Comorbidities			
vascular disease	5	(33.3%)	AMI 1, CI 1, PE 1 , BCS 1, mesenteric vein thrombosis 1 (6.6%)
hematological disease	4	(26.7%)	MPN 4 (26.7%)
diabetes mellitus	3	(20.0%)	

AMI: acute myocardial infarction, BCS: Budd-Chiari Syndrome, CI: cerebral infarction, EHPVO: extrahepatic portal vein obstruction, MPN: myeloproliferative neoplasm, PE: pulmonary embolism.

Table 2. Coagulation inhibitor and causative factors (APS and MPN) for EHPVO patients

Variable	unit (reference range)	Patients tested positive (%)		(range)
Antithrombin III deficiency	% (80-120)	4/13 (30.8%)	89.2 ± 12.9	(65.9-109.0)
Protein S deficiency	% (64-149)	5/12 (41.7%)	77.5 ± 23.2	(46.4-117.0)
Protein C deficiency	% (64-146)	0/12 (0.0%)	90.3 ± 26.2	(64.0-142.0)
D-dimer	ug/mL (<0.5)	13/14 (92.9%)	1.0 ± 0.6	(0.4-2.6)
anti-cardiolipin IgG antibody	U/mL (<10)	0/11 (0.0%)	4.1 ± 1.5	(0-6)
JAK2V617F mutation	(negative)	4 /11 (36.3%)		

APS: antiphospholipid syndrome, EHPVO: extrahepatic portal vein obstruction, JAK: Janus kinase, MPN: myeloproliferative neoplasm.

none of the 4 patients with MPN exhibited thrombo-leukocytopenia. All 4 MPN patients had high counts for both platelets (> 25.5 × 10^4/uL) and white blood cells (> 8.1 × 10^3/uL).

As shown in Table 3, white blood cells and platelets were diminished in EHPVO patients without MPN; however, they were significantly elevated in EHPVO patients with MPN. Furthermore, protein S in EHPVO patients with MPN was significantly lower than in patients without MPN. However, Antithrombin III, Protein C, and D-dimer were not statistically significant between the EHPVO patients with or without MPN. The comparison regarding gender, age at initial symptom, red blood cell counts, history of vascular disease and intestinal bleeding revealed no differences between the EHPVO patients with or without MPN.

In 2 of 4 EHPVO patients with MPN, Hassab's

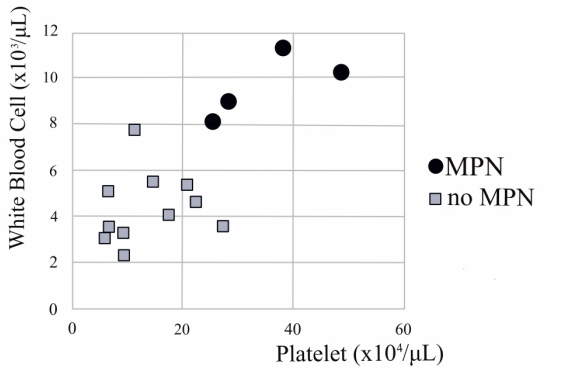

Figure 1. Distribution of platelet and white blood cell counts in EHPVO patients with MPN. Eight of 11 EHPVO patients without MPN (72.3%) exhibited a thrombocytopenic state; however, none of the 4 patients with MPN exhibited thrombo-leukocytopenia. All 4 MPN patients had high counts for both platelets (> 25.5 × 10^4/uL) and white blood cells (> 8.1 × 10^3/uL).

Table 3. Comparison of clinical character of EHPVO patients with or without MPN

Variable	MPN (n = 4)	without MPN (n = 11)	p
Gender (Male/Female)	1/3	6/5	NS
Age at initial symptom			
mean ± SD	54.3 ± 10.0	37.2 ± 18.9	NS
(range)	(42–66)	(5–74)	
BMI kg/m2			
mean ± SD	20.6 ± 1.7	24.6 ± 4.4	NS
WBC ($\times 10^3/\mu L$)			
mean ± SD	9.7 ± 1.2	4.4 ± 1.6	< 0.05
RBC ($\times 10^6/\mu L$)			
mean ± SD	4.4 ± 1.4	4.6 ± 0.4	NS
Platelet ($\times 10^4/\mu L$)			
mean ± SD	35.2 ± 9.2	13.6 ± 7.0	< 0.05
Antithrombin III % (80–120)			
mean ± SD	88.7 ± 10.9	89.4 ± 13.8	NS
Protein S % (64–149)			
mean ± SD	52.9 ± 5.7	89.8 ± 18.4	< 0.05
Protein C % (64–146)			
mean ± SD	84.5 ± 12.1	93.3 ± 30.5	NS
D-dimer ug/mL (< 0.5)			
mean ± SD	1.20 ± 0.55	0.98 ± 0.63	NS
history of vascular disease (yes/no)	1/3	4/7	NS
history of intestinal bleeding (yes/no)	3/1	6/5	NS

BMI: body mass index, EHPVO: extrahepatic portal vein obstruction, MPN: myeloproliferative neoplasm, RBC: red blood cell, WBC: white blood cell.

operation was performed for eradication of esophagogastric varices. Hassab's operation includes splenectomy with devascularization of the upper half of the stomach and distal esophagus. These two patients with MPN caused marked postoperative thrombocytosis of 195 × 10^4 /uL and 222 × 10^4 /uL (*5*), respectively. The increasing platelet counts were thought to be induced by asplenia after splenectomy because hypersplenism preoperatively masked marked thrombocytosis by MPN. The postoperative thrombocytosis due to MPN was treated by administration of cytoreduction therapy by a hematologist.

Various complications due to portal hypertension, including gastropathy, colopathy, biliopathy, intractable ascites, and liver dysfunction, have been reported in adult EHPVO patients (*6*). The rarity and diverse complications of EHPVO make diagnosis difficult and therapeutic strategies diverse.

Nutritional evaluation in our cases revealed that body mass index was within normal limits at 23.4 kg/m^2 with EHPVO. This result is consistent with other reports indicating favorable prognosis in adult patients with appropriate treatment (*7*). Conversely, EHPVO in childhood resulted in retardation of growth in around 50% of pediatric patients, possibly due to reduced portal supply to the liver, resistance to growth hormone and reduced insulin-like growth factor (*6,8*).

EHPVO patients are reported to have a favorable prognosis with a survival rate of 69-86% at 10 years if these portal hypertensive complications are appropriately managed (*7,9-11*). The fatal presentations in chronic EHPVO patients are mainly variceal bleeding and hypersplenism. Considering the frequency and severity of the various portal hypertensive complications, prophylaxis and treatment for esophagogastric variceal bleeding are of particular importance. EHPVO patients with esophagogastric varices show a favorable prognosis if variceal prophylaxis is performed appropriately (*7*). In our study, the proportion of subjects with a history of variceal bleeding with EHPVO was 60% and, likewise, the rate of variceal red color sign was 73.3%, indicating the importance of primary or secondary prophylaxis for variceal bleeding. Furthermore, anti-thrombotic therapy for EHPVO should be administered for portal thrombotic prophylaxis. Long-term anticoagulant therapy also should be administered in EHPVO patients with underlying persistent hypercoagulation (*3,12*). Simultaneous administration for hemorrhagic and thrombotic events complicated treatment for EHPVO by gastroenterologists.

Ectopic varices further complicate the strategy for EHPVO. While ectopic varices account for 1% to 5% of all variceal bleeding in patients with intrahepatic portal hypertension, they account for 20% to 30% of those with EHPVO. In brief, ectopic varices were generally a rare manifestation, but they were a common complication among EHPVO patients (*13,14*). The most difficult aspect is treatment of the ectopic varices due to the lack of standard guidelines because of their rarity. The treatment strategy for hemorrhagic ectopic varices varies by case based on the portal hemodynamics and the site of ectopic varices.

While it is reported that the major causative disease can be detected in the majority of patients with

EHPVO, including MPN, antiphospholipid syndrome, abnormal angiogenesis, and paroxysmal nocturnal hemoglobinuria, none of the risk factors could be identified in a third of patients with EHPVO (*15*). Among these causative diseases, MPN, including chronic idiopathic myelofibrosis, polycythemia vera, essential thrombocythemia, and unclassifiable type (MPN-U), is the most frequent underlying prothrombotic factor for EHPVO with a reported prevalence of 15–30% (*12,16-20*). In the West, MPN has been reported in 58% of patients with EHPVO of unknown etiology, and 57% of these go on to develop an overt MPN during follow up (*20*).

EHPVO in adults is frequently associated with underlying risk factors for hypercoagulation, and the probability of hypercoagulable condition should be considered and screened for (*3,21*). Protein C, Protein S, and antithrombin III deficiencies are associated with the etiology of EHPVO, and detected in 3.9–20.6%, 2–11.8%, and 0–4.1% of EHPVO cases, respectively (*15,22-24*). In this study, Protein S was significantly decreased with EHPBO with MPN. The cause of the reduced levels of Protein S in patients with MPN is unknown; however, the mechanism may be related to the ongoing hypercoagulative condition by MPN that causes the consumption of Protein S.

A close relationship between EHPVO and MPN was also confirmed by the high frequency of a clonal mutation of JAK2V617F, which was present in 16-35% of EHPVO patients (*16,23,25*). Screening for JAK2V617F mutation offers a diagnostic tool for the detection of occult MPN in EHPVO patients (*16,23,26*). JAK2 tyrosine kinase causes cytokine-independent activation of the JAK–STAT pathway, resulting in proliferation of mature myeloid cells (*27*). The JAK2V617F gain-of-function mutation increasing the risk of thrombosis is present in up to 95% of patients with polycythemia vera and in about 50% of patients with essential thrombocythemia and myelofibrosis (*16,28,29*). Furthermore, MPN often sequentially progresses to a fatal condition with an end-stage myelofibrosis or acute myeloid leukemia. These data also suggest the value of screening for JAK2V617F mutation in noninvasive blood sample in EHPVO patients. Patients with splanchnic vein thrombosis, including EHPVO and Budd-Chiari syndrome in adults, show a considerable overlap in etiology; therefore, the JAK2V617F mutation also should be screened for in patients with Budd-Chiari syndrome (*16*).

The present study demonstrated the significant elevation of white blood cells and platelets, and the decrease of protein S in EHPVO with MPN compared to EHPVO without MPN. This hematological difference is very important in understanding the condition of EHPVO with MPN. In particular, we would like to emphasize the importance of platelet counts in EHPVO patients with MPN. The majority of patients with portal hypertension, including EHPVO patients, are usually in an evident thrombocytopenic state due to severe hypersplenism and are, therefore, referred to gastroenterologists (*30*); however, EHPVO patients with MPN have a normal or high platelet count caused by stem cell-derived clonal myeloproliferation despite hypersplenism. The actual high platelet count is effectively masked by hypersplenism to show a normal range, making it difficult for general gastroenterologists to properly understand the underlying state of MPN with hypercoagulation.

Although further research on a large series is required to accumulate more detailed data of EHPVO, our results will encourage gastroenterologists to consider MPN in EHPVO patients.

In conclusion, EHPVO is frequently associated with underlying risk factors for hypercoagulation, causing a dilemma between fatal portal hypertensive bleeding and thrombotic complications. EHPVO patients are usually in an evident thrombocytopenic state due to severe hypersplenism; however, hypersplenic hematologic changes are eliminated in EHPVO patients with MPN. MPN should be strongly suspected in EHPVO patients without thrombo-leukocytopenia.

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**Address correspondence to:*
Tetsuya Shimizu, Department of Gastrointestinal and Hepato-Biliary-Pancreatic Surgery, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8603, Japan.
E-mail: tetsuya@nms.ac.jp

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Correspondence

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Wiskott-Aldrich syndrome: A new synonym mutation in the WAS gene

Yuxin Sun[1,§], Xiaomin Song[2,§], Hua Pan[3], Xiaoxuan Li[4], Lirong Sun[5], Liang Song[6], Fei Ma[1], Junnan Hao[6,*]

[1] Department of Oncology, Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai, China;
[2] School of Basic Medicine, Qingdao University, Qingdao, China;
[3] Department of Pediatric Intensive Care Unit, Affiliated Hospital of Qingdao University, Qingdao, China;
[4] Department of Oncology, Key Laboratory of Cancer Molecular and Translational Research, The Affiliated Hospital of Qingdao University, Qingdao, China;
[5] Department of Pediatric Hematology, Affiliated Hospital of Qingdao University, Qingdao, China;
[6] Department of Emergency Paediatrics, Affiliated Hospital of Qingdao University, Qingdao, China.

SUMMARY Wiskott-Aldrich syndrome (WAS) is a rare X-linked recessive primary immunodeficiency disorder. Mutations in the WAS gene are considered to be the primary cause of WAS. In this work, we report a boy who presented with intracranial hemorrhage (ICH) as an initial symptom and detects a novel pathogenic synonymous mutation in his *WAS* gene. His mother was a carrier of the mutant gene. The mutation, located at position c.273 (c.273 G>A) in exon 2, is a synonym mutation and predicted to affect protein expression by disrupting gene splicing. This study summarizes the diagnosis and treatment process of the patient and expands the genetic spectrum of WAS.

Keywords Wiskott-Aldrich syndrome, synonymous mutation, newborn, hematopoietic stem cell transplantation, intracranial hemorrhage

Wiskott-Aldrich syndrome (WAS) is a rare X-linked recessive disorder which is characterized by thrombocytopenia, microplatelets, eczema, recurrent infections, and an increased risk of autoimmunity and malignance (*1,2*). This disease results from mutations in the *WAS* gene which is located on the short arm of the X chromosome (Xp11.22-p11.23) and contains 12 exons. A wide range of mutations in the *WAS* gene cause WAS protein (WASp) deficiency, leading to immune function defects (*3*). Depending on the type of WAS gene mutation, WAS can ultimately manifest as multiple clinical phenotypes, including classical WAS, X-linked thrombocytopenia (XLT), intermittent X-linked thrombocytopenia (IXLT), and X-linked neutropenia (XLN) (*4,5*).

Intracranial hemorrhage (ICH) is an important cause of neonatal morbidity and mortality (*6*). IHC can be particularly harmful in the neonatal period as this is a critical stage for brain development (*7,8*). Reported cases of WAS that start with ICH are rare. Here, a neonatal patient diagnosed as WAS with a novel gene mutation developed ICH and eventually died of ICH. The aim of this study was to enhance clinical practitioners' awareness of the rare disease onset in WAS patients and to further our understanding of the pathogenesis of WAS. This study was approved by the Ethics Committee of the Affiliated Hospital of Qingdao University School of Medicine. The informed consent has been obtained from the patient's guardian.

A 14-day-old neonatal boy was the first and only child of a Han nationality parents with full-term normal delivery. His mother had a history of thrombocytopenia during the pregnancy. Initially, he was admitted to other hospital because of intracranial hemorrhage, thrombocytopenia, anemia and prolonged activated partial thromboplastin time (APTT). After the surgical removal of intracranial hematoma was used to clear intracranial hematoma, he was transferred to our hospital with eczema. Physical examination showed that the newborn did not have hepatosplenomegaly and lymphadenopathy but had significant eczema on the face and chest. Some rash had ruptured and been infected just looked like the impetigo. The result of hematological examination revealed that the platelet count (PLT, 45.00 $\times 10^9$/L) and plateletcrit (0.04%) were significantly decreased and mean platelet volume (MPV, 7.20 fL) level was at the lower limit of normal complicated with mild anemia (Hb91g/L). Coagulation functions were normal.

Serum IgA, IgM levels were normal except for obvious increased IgE (1,110 IU/mL) and moderately increased IgG (17.50g/L). The percentages of peripheral blood lymphocyte subsets were as follows: CD3+ 66.97%, CD3+CD4+ 60.49%, CD3+CD8+ 5.90%, CD4+/CD8+ 10.25, CD3−CD16+CD56+ 25.79%, and CD3−CD19+ 2.62%. Antinuclear antibodies (ANA) were positive with cytoplasmic (granular) pattern at a 1/100 titer. Antibodies to extractable nuclear antigens (ENA) and complement C3 were negative. Bone marrow biopsy was found to be normal. Evidence of EB virus or CMV virus infection were not found. The case did not find obvious infection except for the impetigo herpetiformis that were cured by systemic antibiotic. The patient was put on intravenous immunoglobulin (IVIG) at the total dose of 2.32 g/kg and the platelet count increased to 61.00 × 10^9/L. Having atypical thrombocytopenia and eczema in a male infant suggested the initial diagnosis of WAS. Then the WAS gene testing confirmed our diagnosis. One month later, the child was re-admitted to the hospital because of thrombocytopenia (PLT25 × 10^9/L). Following IVIG infusion at the dose of 200mg/kg, the child's platelet count raised to 85.00×10^9/L and he was discharged home. Later on, he did not have regular follow-up and eventually died of ICH at the age of 1 year.

As for WAS gene test, DNA extracted from blood samples of the patient were analyzed using next-generation sequencing technologies. The results showed that the patient carries a new hemizygous mutation in the *WAS* gene (Figure 1, b). A G-to-A substitution at position c273 in exon 2 of the *WAS* gene resulted in the mutation (c.273 G>A) (Figure 1, a). This is a silent mutation induced by a base substitution that has no effect on the amino acid it encodes. Meanwhile, Sanger sequencing of the *WAS* genes of the child's parents showed that his mother had a heterozygous mutation in her X chromosome, a G-to-A substitution at position c273 in exon 2 of the *WAS* gene. His father and maternal grandmother revealed wild-type gene sequencing. Therefore, we deduced that the patient's *WAS* C.273 G>A was passed down from her mother, who was an asymptomatic carrier of the mutant gene (Figure 1, c).

The newly discovered mutation did not result in amino acid substitution due to the codon's degeneracy. To explore whether this gene mutation was deleterious, we used online prediction software for preliminary investigation. In Combined Annotation Dependent Depletion (CADD) testing, the mutation site had a RawScore of 3.430166 and a Phred score of 24.8 (Figure 2, b). Both the Spidex and Mutation Taster software prediction results showed that the mutation would result in a splicing change, which would affect the transcript (Figure 2, a).

To further confirm this prediction, we carried out transcriptome sequencing on the patient's and his parents' genes. The results indicated the alteration of the *WAS* gene transcript (Figure 2, c). A novel transcript called ONT.4932.1 was highly expressed in the patient's and his mother's genomes compared to the normal population. WAS-201 was the most highly expressed transcript in the normal population, but it was expressed at a relatively low level in patients. According to the sequence variant interpretation guidelines established by the American College of Medical Genetics and Genomics (ACMG), combined with the clinical manifestations of the child, this variant can be assessed as "pathogenic".

WAS is a rare X-linked recessive disease which threatens the survival and growth of children. Until now, more than 400 different types and sites of mutations in the *WAS* gene have been identified (*9*). The most common type of mutation is missense mutations clustered mainly in exons 1-4, followed by splicing mutations which mainly occur in exons 6-10. The deletion and insertion mutations are relatively rare which

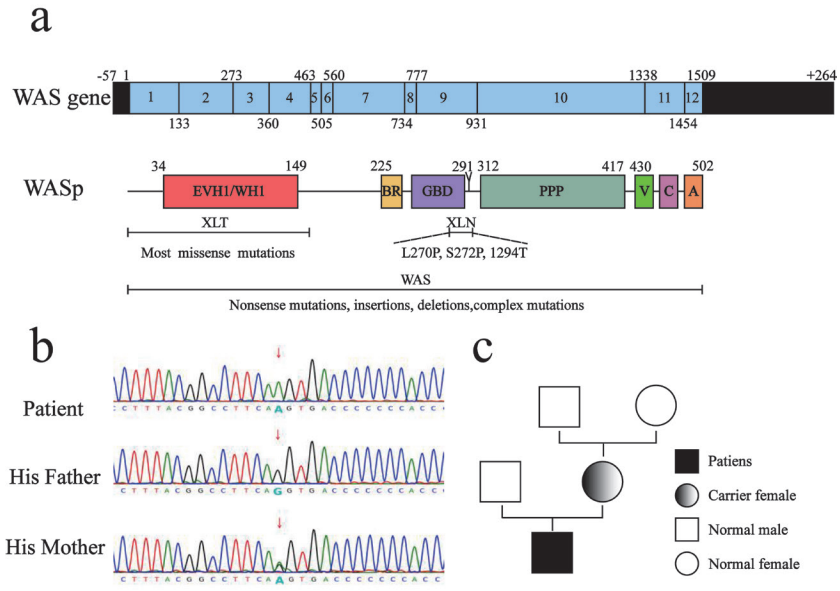
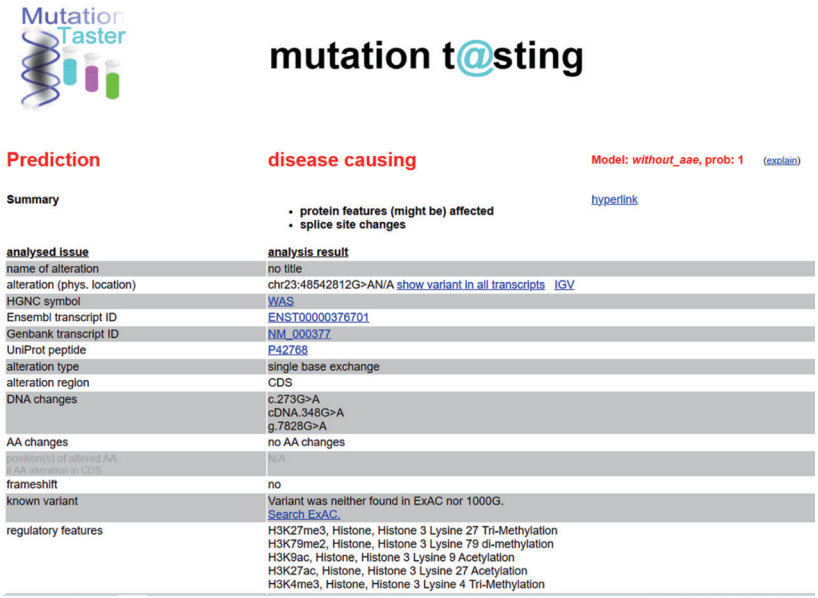


Figure 1. (a) Schematic diagram of the WAS gene and WASp structure. *WAS* gene contains 12 exons and *WAS* c.273 G>A (in patient) occurred on exon 2. WASp consists of five functional domains, namely Ena-VASP Homologous 1 domain (EVH1), base domain (B), guanosine triphosphatase-binding domain (GBD), proline-rich domain (PRD), and verprolin homologous/central hydrophobic C/ acidic domain A domain (VCA). The domain encoded by exon 2 is EVH1. **(b) Sanger sequencing of *WAS* gene in c.273 G>A. (c) Genetic family map of this WAS patient.**

a



b

Chrom	X
Pos	48542812
Ref	G
Alt	A
RawScore	3.430116
PHRED	24.8

c

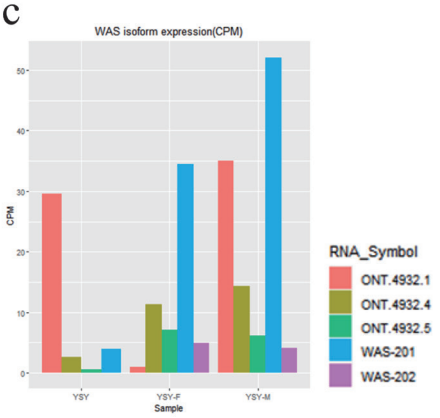


Figure 2. (a) The results of Mutation Taster. (b) The results of CADD testing. (c) The results of transcriptome sequencing.

are distributed in the entire WAS gene (*10,11*). The present study identified a novel synonymous mutation (c.273 G>A), which has not been reported in the HGMD and Clinvar database as a known variant in the general population.

In the process of exploring the pathogenicity of this mutation, next-generation sequencing, SPIDEX and Mutation Taster software as well as transcriptome sequencing were used. At the same time, we traced the origin of the mutation and found that his mother was a carrier of the mutant gene. Therefore, the mutation was considered to be "pathogenic", combined with the child's symptoms and family history. Nonsense mutations had previously been reported at this locus (c273 G>C), but the substituted bases and pathogenicity are different from those in this study (*12*). This finding has important implications for understanding the pathogenicity of mutations in the *WAS* gene and expands the genetic spectrum of WAS.

The treatment depends on the main symptoms, severity and the expected prognosis of WAS (*13*). Hematopoietic stem cell transplantation (HSCT) is currently recognized as the only potentially curative strategy that provides lifelong benefits for typical WAS patients (*14*). As a typical WAS patient, after receiving supportive treatments including antibiotics, immunoglobulins, and platelet transfusion, the clinical symptoms of the patient were temporarily controlled, and humoral immune status was improved. However, the patient eventually died due to recurrent episodes and the lack of curative hematopoietic stem cell transplantation.

Gene therapy for WAS has been rapidly developed in recent years and its therapeutic effect has been widely recognize (*15,16*). Gene therapy involves gene editing of autologous hematopoietic stem cells and reinfusion, which avoids the risks of allogeneic transplantation and effectively overcomes the realistic obstacles of bone marrow matching (*17,18*). With the advancement of treatment methods, the overall survival rate and prognosis of WAS patients has increased. The child might benefit from gene therapy, but don't have the time to receive this therapy and eventually died. Therefore, the effect of gene therapy on WAS caused by this mutation remains to be verified.

In conclusion, the synonymous mutation (c.273 G>C) of exon 2 in the *WAS* gene is pathogenic. Genetic testing

and family history tracing should be done as soon as possible on patients with suspected WAS. In this study, the patient presented with ICH as the first symptom, which is uncommon in patients with WAS. Therefore, infants with ICH as the initial presentation should alert clinicians to the possibility of WAS. Once the diagnosis is established, symptomatic treatment should be combined with early HSCT to improve the prognosis of the patient.

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[§]These authors contributed equally to this work.
**Address correspondence to:*
Junnan Hao, Department of Emergency Paediatrics, Affiliated Hospital of Qingdao University, No. 16 Jiangsu Road, Shinan District, Qingdao, 266000, Shandong Province, China.
E-mail: 15764212800@163.com

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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