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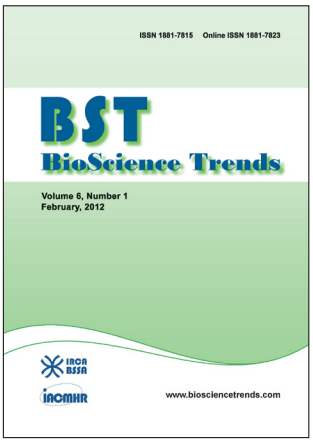

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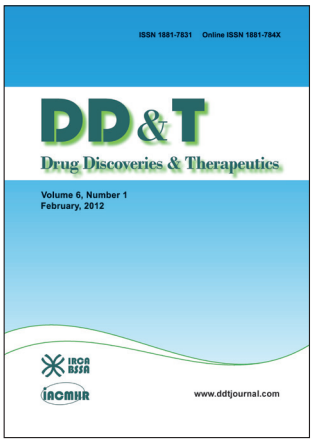

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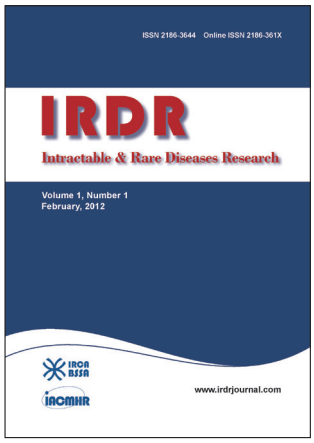

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Marketing of drugs for rare diseases is speeding up in China: Looking at the example of drugs for mucopolysaccharidosis

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Summary **In May 2019, China National Medical Products Administration approved the marketing of an elosulfase alfa injection (brand name: Vimizim) from BioMarin Pharmaceutical for the treatment of patients with mucopolysaccharidosis (MPS) type IVA. This is the first drug to treat MPS in China, and it has ended the "dearth of medicines" to treat MPS in China, a situation that has persisted for many years. One can reasonably say that the drug has benefited from the continuous reform of the drug review and approval system in China and the increasing attention paid to rare diseases. At present, China has implemented a series of preferential policies for the review and approval of drugs for rare diseases, mainly including priority review and approval, accelerated review and approval, special review and approval (mainly simplified review and approval), data protection, and communication. Moreover, China now has a specific reference for the review and approval of drugs for rare diseases with the creation of China's First List of Rare Diseases and the publication of two batches of the List of Overseas New Drugs Urgently Needed in Clinical Settings. Drug review and approval has been significantly accelerated, as has marketing. The two batches of lists of new drugs, issued in November 2018 and May 2019, include 43 drugs for rare diseases (58.1% of all drugs in the lists), 37 of which were included in China's First List of Rare Diseases. The lists also include three other drugs for MPS. As of July 1, 2019, four drugs for rare diseases from the first batch of new drugs have been approved for marketing. In order to further improve the review and approval of drugs for rare diseases in China, a special department should be established for the evaluation of drugs for rare diseases, research on and management of drugs in the post-approval phase should be enhanced, international cooperation in research on use of drugs to treat rare diseases should be enhanced, and the incentive policy for marketing drugs for rare diseases should be improved.**

Keywords: Rare disease, drugs for rare diseases, drug review and approval, China, mucopolysaccharidosis

1. Introduction

Rare diseases are a group of diseases with very low incidence and prevalence. Currently, less than 10% of patients with rare diseases have access to specific treatments (*1*). The limited number of patients means that limited attention is paid to those diseases, and few clinical trials of drugs are conducted. This reality has greatly hindered the timely marketing of drugs for rare diseases, and it has delayed effective and timely treatments for patients with rare diseases. The situation is even worse in China. Statistics indicate that prior to December 2018 only 83 drugs for rare diseases had been marketed in China (according to China's First List

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of Rare Diseases, hereinafter referred to as the Chinese Rare Diseases List, or CRDL); these drugs account for only 51% of orphan drugs around the world (*2*).

Fortunately, continuous reform of the drug review and approval system and increasing public attention paid to rare diseases has accelerated the review and approval process for drugs to treat rare diseases in China. In May 2019, the National Medical Products Administration (NMPA) authorized the marketing of an elosulfase alfa injection (brand name: Vimizim) from BioMarin Pharmaceutical for the treatment of mucopolysaccharidosis (MPS) type IVA. This is the first drug to treat MPS in China, having ended the "dearth of medicines" to treat MPS in China for many years. The drug was included in *the List of the First Batch of Overseas New Drugs Urgently Needed in Clinical Settings* (hereinafter referred to as the First New Drug List, FNDL) issued by the Center for Drug Evaluation (CDE) of the NMPA in November 2018 and authorized for marketing in May 2019, indicating that the drug review and approval process has accelerated (*3*).

2. Mucopolysaccharidosis

2.1. *Basic features*

MPS is a complex, progressive, and multi-system lysosomal disease caused by a lack of enzymes that degrade glycosaminoglycans. Mucopolysaccharides that cannot be completely degraded are stored in lysosomes, which leads to facial abnormalities, nervous system involvement, skeletal deformities, enlarged liver and spleen, heart disease, corneal opacity, *etc*. (*4*). MPS is classified into 7 types that involve 11 lysosomal enzymes encoded by 11 genes (I, II, IIIA, IIIB, IIIC, IIID, IVA, IVB, VI, VII, and IX).

Table 1. Incidence of different types of MPS

Type	Asian (*10-16*)	Global (*10*)
I	1/100,000	1/100,000
II	1/100,000	1/140,000-160,000
III	A:1/100,000	1/70,000-90,000
	B:1/200,000	
	C:1/1,500,000	
	D:1/1,000,000	
IV	A:1/201,000	1/200,000
	B:1/76,000-640,000	
VI	1/240,000-400,000	1/240,000-300,000
VII	1/400,000	<1/250,000
IX	Only 4 reported cases	Extremely rare

MPS, mucopolysaccharidosis.

2.2. *Epidemiological features*

The prevalence of MPS is approximately 1/100,000 (*4*). The incidence of MPS is shown in Table 1. In Asian countries like South Korea and Japan, about 50% of patients have MPS type II, while the incidence of MPS type I is higher than that of MPS type II in Western countries (*5*). Prior to January 31, 2019, 176 patients with MPS were identified in Taiwan, China (*6*). Although there is a lack of epidemiological data on MPS in mainland China with only individual studies of clinical cases, the disease has been included in many rare disease catalogues in China, including the CRDL (*7*), *the List of Major Rare Diseases in Shanghai (2016 edition)* from the former Shanghai Municipal Health and Family Planning Commission (now called Shanghai Municipal Health Commission) (*8*) and *China's Rare Diseases Reference List (Revised Edition)* from a non-profit organization (*9*).

2.3. *Treatments and drugs*

The most common treatments for MPS are hematopoietic stem cell transplantation and enzyme replacement therapy (*17*). Many of the drugs for enzyme replacement therapy are already on the market in the US, the EU or Japan, and some came on the market in the US 10 years ago, including laronidase (2003), idursulfase (2006) and galsulfase (2005). Some drugs have also been marketed in the US in recent years, including elosulfase alfa (2014) and vestronidase alfa (2017). Table 2 shows the global

Table 2. The status of global marketing of drugs for MPS (*2,3,18-21*)

General name	Brand name	Indication	Marking status			
			USA	EU	Japan	China
Laronidase	Aldurazyme	MPS I	2003/04	2003/09	2006/10	Included in the SNDL (2019/05).
Idursulfase	Elaprase	MPS II	2006/07	2007/01	2007/10	Included in the SNDL (2019/05).
Dursulfase beta	Hunterase	MPS II	South Korea (2012/07)			Pharmaceutical companies signed an agreement on exclusive licensing in China (2019/01). An application for marketing approval has been received by the NMPA (2019/07).
Elosulfase alfa	Vimizim	MPS IV	2014/02	2014/04	2014/12	Included in the FNDL (2018/11). Approved (2019/05).
Galsulfase	Naglazyme	MPS VI	2005/05	2006/01	2008/03	—
Vestronidase alfa	MepseVII	MPS VII	2017/11	2018/06	—	Included in the FNDL (2018/11).

MPS, mucopolysaccharidosis; FNDL, First New Drug List, *the List of the First Batch of Overseas New Drugs Urgently Needed in Clinical Settings*; SNDL, Second New Drug List, *the List of the Second Batch of Overseas New Drugs Urgently Needed in Clinical Settings*.

marketing of drugs for MPS.

However, these drugs have yet to be approved in China. Hence, symptomatic treatment is often provided in China, with the goal of treating respiratory and cardiovascular complications, deafness, hydrocephalus, along with surgery and rehabilitation in order to improve the quality of life of patients with MPS (*4*). The review and approval of drugs for rare diseases in China has been significantly accelerated by the reform of the drug review and approval system and the introduction of policies on rare diseases in recent years. Elosulfase alfa for the treatment of MPS type IVA and vestronidase alfa for the treatment of MPS type VII were included in the FNDL (*3*). Laronidase for MPS type I and idursulfase for MPS type II were included in *the List of the Second Batch of Overseas New Drugs Urgently Needed in Clinical Settings* (hereinafter referred to as the Second New Drug List, SNDL) issued in May 2019 (*18*). Elosulfase alfa, which treats MPS type IVA, was officially approved by the NMPA in May 2019.

3. The development of review and approval policies of drugs for rare diseases in China

3.1. *Early stage (Before 2015)*

Provision for Drug Registration, which were formulated in accordance with the *Pharmaceutical Administration Law of the People's Republic of China*, are the fundamental policy for drug review and approval. The *Provision* mentioned how new drugs for rare diseases with obvious clinical advantages could be specially approved (*22*). The *Regulations to Manage the Special Approval of New Drugs* were issued in 2009 (*23*), and they included three main mechanisms: dynamic supplementation of materials through multiple channels, multi-channel communication, and a reduced approval time. However, a study indicated that the average time for review of drugs for rare diseases was 351 days (*24*).

3.2. *The reform of drug review and approval (2015-)*

The *Opinion on Reform of the Review and Approval System of Drugs* and Medical Devices was published by the State Council in August 2015 (*25*), marking the beginning of a new round of reform of the drug review and approval system in China. The reform is of great significance since it seeks to improve the quality of review and approval, reduce the backlog of applications for registration, improve the quality of generic drugs, encourage research and development of new drugs, and improve the transparency of drug review and approval. Accelerating the review and approval of innovative drugs for rare diseases was mentioned in the *Opinion*. Since then, a number of specific policies on drug review and approval have been introduced. In October 2017, the Central Office of the Communist Party of China and the General Office of the State Council issued their *Opinion on Further Reform of the Review and Approval System and Encouraging Innovation in Drugs and Medical Devices*, signaling further reform (*26*). One specific section mentioned supporting the development of drugs and medical devices for rare diseases. The aforementioned reform has created a good external policy environment to accelerate the marketing of drugs for rare diseases in China.

3.3. *Publication of the CRDL (2018-)*

Although the review and approval of drugs for rare diseases has benefited from a series of policies, these policies cannot be effectively implemented since China lacks a clear definition or scope of rare diseases. Social security for patients with rare diseases in China has a clear and priority range, as identified by the publication of the CRDL in May 2018 (*27*). Moreover, arrangements to accelerate the approval of overseas new drugs were made by Premier Li Keqiang at the Executive Meeting of the State Council on June 20, 2018. Simplification of the marketing requirements for drugs to treat rare diseases has been proposed, and applications for marketing approval can be submitted with research materials from overseas. Regulatory authorities should conclude review of an application within three months (*28*). In October 2018, *Procedures for the Review and Approval of Overseas New Drugs Urgently Needed in Clinical Settings* were issued by the NMPA and National Health Commission (*29*). Drugs for rare diseases that have been marketed in the US, the EU, or Japan for ten years can directly receive marketing approval and would be included in special channels for review and approval. Since the *Procedures* were issued, the review and approval of drugs for rare diseases in China has really sped up. Material requirements for drugs in different stages are shown in Table 3.

In general, the current preferential policies for the review and approval of drugs for rare diseases in China include prior review and approval, accelerated review and approval, special review and approval (mainly simplified review and approval), and data protection and communication, as shown in Table 4.

4. Recent approval and review of drugs for rare diseases in China

The FNDL includes a total of 48 types of drugs, with 25 drugs for rare diseases (not including rare tumors); 20 of those drugs are to treat diseases in the CRDL. Prior to July 1, 2019, 4 of the 20 drugs had been approved for marketing, 2 were under review, 4 were preparing for application, 6 were not scheduled for marketing approval, and 4 had no contact (*35*). The SNDL includes a total of 26 types of drugs, with 18 drugs for rare

Table 3. Material requirements for an application for marketing approval of overseas new drugs urgently needed in clinical settings (*29*)

Drug status	Application for marketing approval
Drugs that have not yet been submitted for clinical trials or marketing approval	Submit an application for marketing approval.
Drugs that have been submitted for clinical trials but that have not completed technical review	Adjust the clinical trial application to the application for marketing approval. Supplement all research materials from overseas and supporting materials indicating no ethnic differences in action.
Drugs undergoing clinical trials	Submit an application for marketing approval and continue the clinical trial. After completing the clinical trial, submit a research report in the form of a supplementary application.
Drugs that have submitted for marketing approval	Supplement all research materials from overseas and supporting materials indicating no ethnic differences in action.
Drugs that have been marketed in Japan or Chinese Hong Kong, Macau, Taiwan with abundant cases	Provide research reports on drug utilization in the aforementioned countries or regions and perform relevant analysis; may not need to provide research materials on ethnic differences in action.

Table 4. The main preferential policies for the review and approval of drugs for rare diseases in China

Type of Policy	Main contents
Prior review and approval	Establish a special channel of review and approval for drugs that have been included in the List of New Drugs (*27*). Examine classified review and approval of drugs for rare diseases, children, and the elderly (*30*).
Accelerated review and approval	CDE should complete the technical review of drugs in the List of New Drugs within 3 months of acceptance (excluding the time taken for the applicant's supplementary materials), and NMPA should make a decision in 10 working days after receiving the review materials from CDE (*27*).
Specialized/Simplified review and approval	When applying for a clinical trial, the applicant can apply to reduce the number of subjects or to receive an exemption from clinical trials (*22,31*) . An application for marketing approval of drugs that have been listed overseas and that are believed to have no ethnic differences in action can be submitted with clinical trial data from overseas (*32*). Still soliciting opinions: real-world data from natural disease cohorts could be used as external controls, and external controls are mainly used for non-random single-arm trials, which can be historical or parallel (*33*).
Data Protection	A certain period of protection should be provided to data acquired by the applicant and undisclosed trial data. During the period of data protection, an application for similar marketing approval by another applicant should not be approved except in situations where the applicant obtained the data or the applicant received the consent of a company marketing the drug (*26*). Still soliciting opinions: 6 years of data protection should be provided starting from approval in China (*34*).
Communication	CDE should establish a mechanism for communicating with applicants to enhance its guidance of drug development (*29,32*).

CDE, Center for Drug Evaluation.

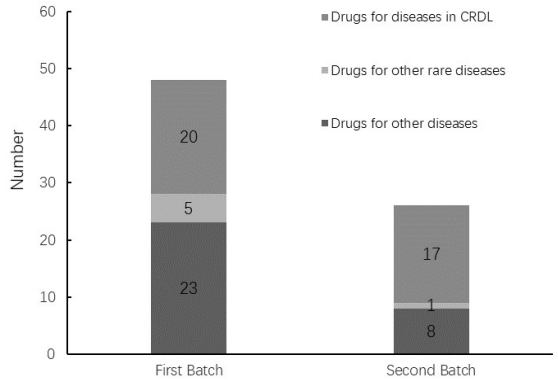

Figure 1. Drugs on the two lists of the First Batch and Second Batch of Overseas New Drugs Urgently Needed in Clinical Settings. CRDL, China's First List of Rare Diseases

diseases; 17 of those drugs are to treat diseases in the CRDL. Types of drugs on the two lists are shown in Figure 1.

5. Discussion and Suggestions

Despite the accelerated approval of drugs for rare diseases, the review and approval process still faces many challenges in China. There is still much work to do in order to further improve the marketing of drugs for rare diseases.

5.1. *Establishing a specialized department for the review and approval of drugs for rare diseases*

The current and future workload for the review and approval of drugs for rare diseases is relatively heavy, since only about half of the drugs for rare diseases are on the market in China. A specialized department in the CDE needs to be established to review and approve drugs for rare diseases. The Office of Orphan Products Development (OOPD) has been set up in the US FDA (*36*), and the Committee for Orphan Medicinal Products (COMP) has been set up in the EMA (*37*). This guarantees the effective review and approval of drugs for rare diseases and it also accelerates expert review and approval of those drugs. These benefits will play an important role in promoting the development and marketing of drugs for rare diseases in China.

5.2. *Enhancing the research and management of drugs for rare diseases after marketing*

Rapid or special approval of drugs for rare diseases is currently available. However, research on and management of rare disease drugs should be enhanced considering the possible risks of drug use and the great value of patient research. Both the US and the EU have implemented post-approval management for drugs to treat rare diseases (*38,39*). The FDA issued guidelines on post-marketing research and clinical trials in 2011. In addition, pharmaceutical companies are also obliged to inform doctors about information on drug usage and risks and to conduct risk management (*40*). Another urgent task is to establish and improve post-marketing research and management systems for drugs to treat rare diseases, such as enhancing physician training, establishing registries of drugs use, and collecting real-world data.

5.3. *Enhancing international cooperation in research on rare diseases*

With the accelerated marketing approval of drugs to treat rare diseases in China and the establishment of rare disease registries and patient organizations (*41*), information on patients with rare diseases and their medications in China has been fleshed out further. International cooperation on rare diseases, and especially on drugs used, should be coordinated. This will greatly promote the development, launch, and utilization of those drugs.

5.4. *Improving the incentive policy for marketing approval of drugs for rare diseases*

Although the review and approval of drugs for rare diseases has accelerated, many drugs for rare diseases still have yet to be approved, scheduled, or contacted for marketing in China despite their appearance in the List of New Drugs. In addition to the continuous improvement of the review and approval of drugs for rare diseases, appropriate incentive policies should be formulated to attract pharmaceutical companies. Detailed rules for the implementation of polices, like data protection for patients with rare diseases, need to be issued. Exclusivity of drugs for rare diseases could be implemented. More drugs for rare diseases need to be covered by social insurance.

6. Conclusion

The review and approval of drugs for rare diseases has been markedly accelerated in China. This was initially the result of reform of China's drug review and approval system and the publication of CRDL, though it could not have been achieved without further reform of the health care system, continued reform of social welfare, and optimization of the administrative review and approval system. Given the accelerated introduction of drugs for rare diseases, more patients with rare diseases will presumably have access to those medicines, and those medicines will gradually become more available to patients with rare diseases in China (*42*). However, this is just the first step to improving drug accessibility for patients with rare diseases, since affordability and rational use of medicines are essential as well. Medical care for patients with rare diseases in China still has a long way to go.

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Review

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Genetics of hereditary hearing loss in east Iran population: A systematic review of *GJB2* mutations

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Summary **Mutations in the *GJB2* gene are the most common cause of pre-lingual hearing loss (HL) worldwide. Previous studies have shown the frequency of *GJB2* mutations to be 16% in Iran, but varies among different ethnic groups. Here, we have reviewed results from previous published mutation reports to provide a comprehensive collection of data for *GJB2* mutations and HL in eastern Iran. We conducted a systematic literature review of PubMed, Google Scholar, Web of Science, and Science Direct databases for articles published before March, 2019. The literature search was performed by 2 independent researchers. The primary data of these studies including the number of samples, allelic frequency, and so on were extracted. Six studies involving 812 unrelated families from four different eastern provinces were included and analyzed for the type and prevalence of *GJB2* mutations. A total of 19 different genetic variants were detected. *GJB2* mutations were 8.8% in the studied eastern provinces, which was lower than that reported in northern populations of Iran. Moreover, a gradient in the frequency of GJB2 mutations from north to south Iran was observed. c.35delG was the most frequent mutation, accounting for 48.5% % of the populations studied. However, this mutation was absent in the Baluchi population. This review shows that particular rare mutations are frequent in some Iranian ethnic groups, and should be considered for genetic counselling.**

Keywords: Iranian population, genetic counseling, *GJB2*, non-syndromic hearing loss

1. Introduction

Hearing Loss (HL) is the most common sensory disorder, affecting 1 in every 500-1,000 newborns (*http://hearing.screening.nhs.uk/nationalprog*). It is estimated that 50-70% of HL is related to genetic causes. Almost two thirds of cases include non-syndromic forms (NSHL), since hearing impairment is the only sign. HL is divided into DFNB autosomal recessive (80%), DFNA autosomal dominant (17%), X-linked (2-3%) and mitochondrial type (> 1%) (*1*). Autosomal recessive NSHL (ARNSHL) is highly heterogeneous, with over 60 identified causative genes (*http://hereditaryhearingloss.org/*). Remarkably, defects in one locus (DFNB1) accounts for up to 50% of the etiology in many western populations (*2-4*), which makes this the most common cause of NSHL (*5*). The DFNB1 locus containing *GJB2* and *GJB6* genes encode connexin26 (Cx26) and connexin30 (Cx30) respectively. Connexins are a kind of gap junction protein involved in inner ear homeostasis *via* recycling of potassium ions (*6*). To date, more than 100 pathogenic mutations in the *GJB2* gene have been identified resulting in ARNSHL (*7*). The prevalence of *GJB2* mutations varies among different populations (*8-16*). In Caucasians, c.35delG is the most

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common mutation with carrier frequency as high as 2-4% (*17*). However, c.235delC, c.167delT and c.71G>A are the most frequent mutations in the Japanese (*18*), Ashkenazi Jewish (*19*), and Indians (*20*), respectively. The Iran population is genetically heterogeneous and a mixture of several ethnicities; consanguineous pedigrees affected with ARNSHL have helped to identify many HL associated genes. Over the past 15 years, tremendous amounts of epidemiologic data have been collected on the Iranian population in order to determine the mutation spectrum and frequency of *GJB2* mutations (*21-30*). It was shown that the mutation frequency of *GJB2* varies between 0 and 35% among different regions of Iran and c.35delG is the most common mutation reported (*31,32*). In this paper, we summarized the published data on the frequency and profile of the *GJB2* gene mutations in 812 unrelated families from 4 different provinces; namely Khorasan, Sistan & Baluchestan, Kerman and Hormozgan in east Iran compared to other parts of this country.

2. Methods and Analysis

2.1. *Publication search*

A systematic literature review of PubMed, Google Scholar, Web of Science, and Science Direct databases was conducted in English for articles published before April, 2019. The following keywords and medical subheadings were used simultaneously in each set: ("hearing loss" or "deafness" or "hearing impairment") and ("GJB2") or ("connexine26") or ("35delG") and ("Iran"). Alternative spellings were also considered. The literature search was performed by 2 independent researchers.

2.2. *Inclusion criteria and data extraction, data analysis*

Eligible studies included in our review met the following inclusion criteria: *i*) performed on non-syndromic HL subjects, *ii*) described ethnicity of tested subjects, and *iii*) detected all *GJB2* mutations. Studies were excluded if HL was a result of environmental factors such as infection, trauma, rubella, meningitis, mumps, ototoxic drugs and premature birth. The flow diagram of the selected eligible studies is shown in Figure 1. The necessary data were extracted from the final eligible articles as follows: first author, publication year, subject ethnicity and number of cases. The frequency and mutation type of *GJB2* were extracted from relevant studies and categorized, corresponding with geographical boundaries. *In silico* analyses were also performed with available software tools to predict pathogenicity of the mutations.

3. Results

A total of 6 studies comprising 812 unrelated families from 4 provinces were included for analysis. The

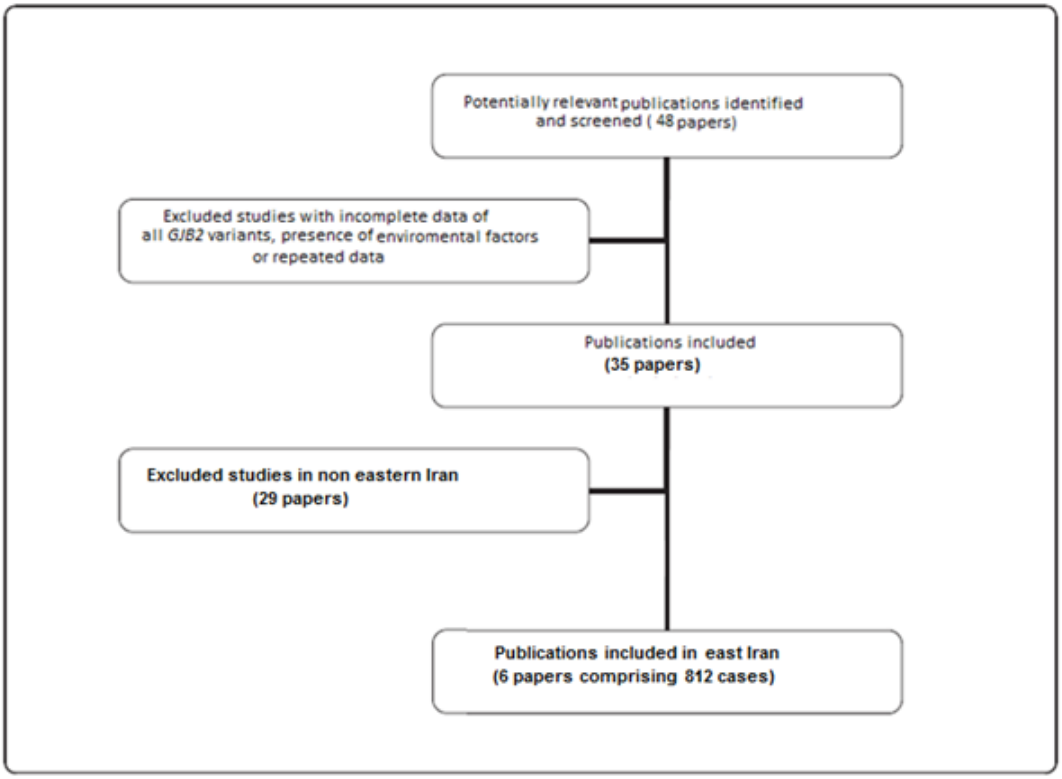

Figure 1. Flow chart of review process.

Table 1. The frequency of consanguinity in different provinces of east Iran

Province	Sistan & Baluch	Kerman	Khorasan	Hormozgan
Consanguinity	258	87	212	75
Non-consanguinity	46	16	84	46
Total	292	103	296	121

groups studied consisted of 296 families from Khorasan province (36.45%), 292 families from Sistan & Baluchestan (35.9%), 121 families from Hormozgan (14.9%) and 103 families from Kerman province (12.6%). Among these families, 78.7% reported parental consanguinity while in 21.3% close consanguinity was denied (Table 1). The *GJB2* mutation allele frequencies of each studied group included 13.7%, 8.4%, 7%, 6.3% of total studied families (n = 812) of Khorasan, Hormozgan, Kerman, and Sistan & Baluchestan provinces, respectively. A total of 19 different variants were identified, 14 of which were reported as pathogenic. These include: c.-23+1G>A, c.35delG, c.71G>A, c. 336G>T, c.167delT, c.235delC, c.29delT, c.358-360delGAG, c.238G>A, c.427C>T, c.269T>C, c.511G>A, c.229T>C, c.465T>A. The *GJB2* mutation spectrum and frequency revealed in this review is listed in Table 2. In the studied populations, c.35delG was the most frequent mutation accounting for 48.5% of the populations studied. The highest rate of c.35delG mutation was detected in Khorasan province with an allele frequency of 10.1% while we did not find any c.35delG mutations in Sistan & Baluchestan (Figure 2). A specific combination of *GJB2* mutation types and frequencies were found in different studied provinces (Table 2). A higher *GJB2* mutation diversity (9 types) was observed in Khorasan province while the lowest diversity was identified in Sistan & Baluchestan (4 types).

4. Discussion

The identification of genes causing non-syndromic hearing Loss (NSHL) has partially resolved the puzzle of clinical and genetic heterogeneity of HL (*33-35*). Among these genes the gene with the most significant impact on population genetics and genetic counselling is the *GJB2* gene with the mutation c.35delG that accounts for the majority of mutations in deaf Caucasians (*12*). Studies published so far have reported the differences in frequency of the mutation in different populations, even from neighboring countries. The Iranian population is composed of many different ethnic groups. According to this fact, it is necessary to discuss ethnic specific data. This study reviews the prevalence and type of the *GJB2* gene mutations of 812 deaf families from four different provinces of this country. Here, the most consistent finding was the reduction of *GJB2* mutation frequencies of north to south Iran. Our data showed a north to south gradient among Iranian populations with a *GJB2* mutation frequency of 13.7% for Khorasan province and 6.3% for Sistan & Baluchestan province. In 2007, Hashemzadeh *et al*. stated that the frequency of *GJB2*-related HL is in 14.6% of deaf families (130 of 890 families). They also found the highest percentage of *GJB2* related HL in the north and northwest regions of Iran (27%), while it was less than 4% in the Southeast region (*36*). The findings of our study shows that the contribution of *GJB2* mutations to ARNSHL is 13.7% in Khorasan province, which is similar to the presented data from Semnan province (*37*). Bazazzadegan *et al*. screened 111 ARNSHL families from Semnan province in center Iran for *GJB2* mutations. They reported that *GJB2* mutations were detected in 11.5% of the ARNSHL families studied.

Another finding of this study was the mutation spectrum of southeast Iran, which was different from those of other Iranian population regions. Two hundred and ninety two unrelated Baluchi deaf families were reviewed, with a frequency of 6.2% for *GJB2* gene mutations. Interestingly, c.71G>A was the most frequent *GJB2* mutation, while c.35delG was absent in this ethnicity. Results obtained for the carrier frequency of c.71G>A mutation was 70% in the Baluchi population whereas 4.2% in the rest of eastern provinces. However, the Baluchi population is ethnically distinct from the rest of Iran. Besides, 121 unrelated ARNSHL families from Hormozgan province were reviewed, with a frequency of 8.4% for *GJB2* gene mutations. This rate of *GJB2* mutation has been reported in some populations of the south of Iran like Khuzestan province (*37*). In the study performed by Bazazzadegan *et al*. (*37*) on 103 ARNSHL families indicated that *GJB2* related HL accounted for 7% in Kerman province. This is about one third of the frequency of the *GJB2* mutations in Isfahan province. In the previous study, we showed that *GJB2* mutations explain the cause of ARNSHL in 22.5% of patients from Isfahan province in the center of Iran (*38*). In addition, Davarnia *et al*. (*39*) screened 50 NSHL families from Ardebil province in northwest Iran for *GJB2* mutations. They reported that *GJB2* mutations were found in 26% of the NSHL families studied. On the basis of these results, it can be concluded that the frequency of *GJB2* mutations decreases gradually both west to east and north to south (Figure 3), drawing the migration pathway of the initial founders.

In our studied populations, the most common mutation was c.35delG, accounting for 48.5% of *GJB2* mutations. The c.35delG mutation (deletion of guanine in position 30-35; rs80338939) is the most common

Table 2: GJB2 mutations, their frequencies and *in silico* analyses in seven provinces of Iran*

Mutations	Province (*ref.*)								Mutation type	Classification	Functional effect	
	Kerman (*37*)	Hormozgan (*44*)	Hormozgan (*45*)	Sistan & Baluch (*37*)	Sistan & Baluch (*46*)	Sistan & Baluch (*47*)	Khorasan (*41*)	Khorasan (*37*)			Mutation Taster	SIFT
c.29delT	.	2 (6.25)	.	.	.	.	.	.	Frameshift	T	Disease causing	NA
c.35delG	4 (1.94)	.	3 (1.42)	.	.	.	25 (11.16)	33 (8.96)	Frameshift	T	Disease causing	NA
c.71G>A	2 (0.97)	.	2 (0.95)	12 (5.5)	2 (1.19)	10 (5)	.	.	Missense	NT	Disease causing	Damaging
c.167delT	.	.	.	2 (0.92)	2 (1)	2 (1)	.	1 (0.27)	Frameshift	T	Disease causing	NA
c.229T>C	.	.	.	.	.	.	2 (0.89)	.	Missense	NT	Disease causing	Damaging
c.235delC	.	.	.	.	.	.	2 (0.89)	.	Frameshift	T	Disease causing	NA
c.238G>A	.	2 (6.25)	.	.	.	.	.	.	Missense	NT	Disease causing	Damaging
c.269T>C	.	.	.	.	.	.	1 (0.45)	1 (0.27)	Missense	NT	Disease causing	Damaging
c.358-360delGAG	4 (1.94)	.	.	.	.	.	1 (0.45)	2 (0.54)	Frameshift	T	Disease causing	NA
c.336G>T	.	.	.	.	1 (0.5)	1 (0.5)	.	1 (0.27)	Missense	NT	Disease causing	Damaging
c.427C>T	1 (0.48)	.	.	.	.	.	.	.	Missense	NT	Disease causing	Damaging
c.465T>A	1 (0.48)	.	.	.	.		.	.	Missense	NT	Disease causing	Damaging
c.551G>C	.	.	.	.	.		.	2 (0.54)	Missense	NT	Disease causing	Damaging
c.23+1G>A	.	.	.	.	1 (3.2)	1 (3.2)	.	2 (0.54)	Splice site	NT	Disease causing	NA
c.79G>A	.	.	6 (2.85)	.	1 (0.59)	.	8 (3.57)	.	Missense	NT	Polymorphism	Tolerated
c.101T>C	.	.	.	.	.	.	1 (0.45)	.	Missense	NT	Polymorphism	Benign
c.341A>G	.	.	.	.	1 (0.59)	.	3 (1.34)	.	Missense	NT	Polymorphism	Benign
c.380G>A	2 (0.97)	.	4 (1.9)	4 (1.85)	2 (1.19)	4 (2)	3 (1.34)	.	Missense	NT	Polymorphism	Benign
c.457G>A	.	.	3 (1.42)	.	8 (4.76)	.	3 (1.34)	.	Missense	NT	Polymorphism	Benign
Normal	192	28	192	198	154	182	175	326				
Total	206	32	210	216	168	200	224	368				

*The pathogenic mutations and benign variants were separated in the two parts. The mutations were arranged in numerical order. T, Truncated protein; NT, Non-Truncated protein; NA, Not Available.

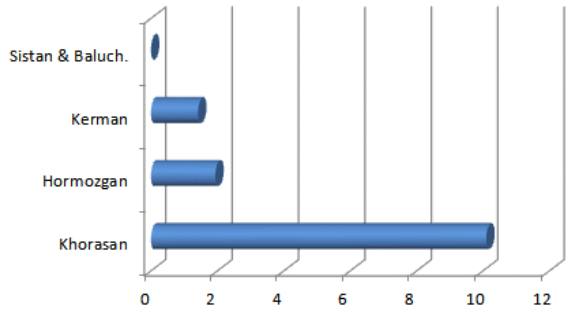

Figure 2. The allele frequency of c.35del G mutation in different provinces of east Iran.

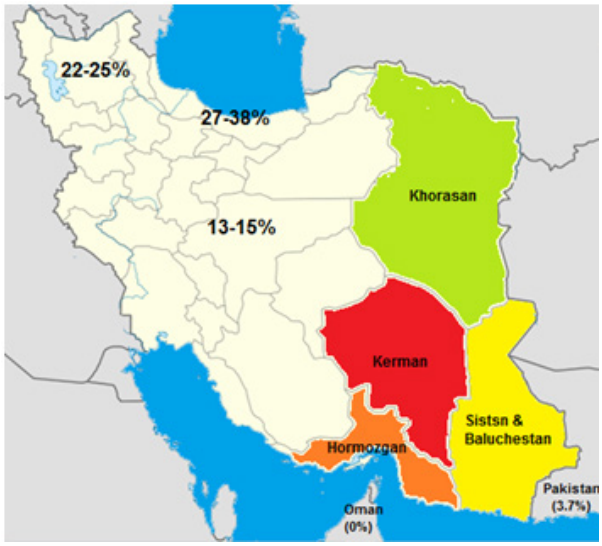

Figure 3. The prevalence of *GJB2*-related mutations in different regions of Iran (west 22-25%, north 27-38% (*32,41*), center 13-15% (*32,37*)). Four eastern provinces (Sistan & Baluchestan, Hormozgan, Kerman and Khorasan) are shown in the map.

mutation in many world populations as well as many countries in the Middle East such as Turkey, north and northwest of Iran (*40*). The study of the geographical distribution of the *GJB2* mutations showed less allelic heterogeneity in the east compared to the north of Iran. The four most frequent mutations of the *GJB2* gene in the west of Iran, namely, c.35delG, c.71G>A, c.358_360delGAG and c.167delT are responsible for ~79.8% of all pathogenic alleles in east Iran (Table 2). The c.35delG mutation, which is the most common (up to 85%) among northern regions (*41*), makes up 48.5% of *GJB2* mutations in the eastern populations. The c.71G>A, c.358_360delGAG and c.167delT are the second, third and fourth most common mutations, with a sum of 20.9%, 5.2% and 5.2% of all pathogenic alleles. The p.Trp24*, a nonsense mutation is the result of c.71G > A transition, changing the TGG codon for Trp residue to a stop codon, which leads to a truncated protein with probably no functional properties. *In silico* analyses are consistent with the pathogenicity of the mutation (Table 1). Sistan & Baluchestan province is located on the western border of Pakistan and is populated mainly by Sistani & Baluchi ethnicities. The c.71G > A mutation is the most common mutation in Pakistan and Indian populations. The rate of carriers of c.71G > A mutation is 4.08% in the Pakistan population (*42,43*). This mutation shows a high frequency in the Baluchi group, where the population is related to neighboring Pakistan. This review showed a particular combination of *GJB2* mutations diversity in different provinces of east Iran. A higher *GJB2* mutations diversity (9 types) was detected in Khorasan province, suggesting the co-existence of several different ethnic groups and immigrations to big cities such as Mashhad during the last century. In addition, historical background like occurrence of different wars with foreign nations, immigration, and location of the route of the Silk Road could support this diversity. In contrast with the high diversity of Khorasan province, we found a very low rate of diversity in some populations such as the Baluchi population who are probably isolated with cultural and geographical barriers. The limitation of this study is a small number of studies especially for Kerman province (103 families). Therefore, more screening programs in the east population are, warranted.

5. Conclusion

The critical and specific position of Iran and the existence of various ethnic groups with different cultures suggest high heterogeneity throughout Iran but specific intra ethnic traditions such as intragroup marriages may give rise to a high homogeneity in some loci and mutations within groups. Our study revealed a frequency of 8.8% for *GJB2* mutations, relatively low compared to other studies of central populations with estimated frequencies between 13-15%. Referring to the *GJB2* mutations, one section of this cohort had the c.35delG mutation, so this mutation is the most common mutation that is tested first. In studied populations, specific mutations are common, which are detected in each group; for example, the frequency of the c.71G>A shows a high rate in Sistan & Baluchestan province, accounting for 70% of the mutant alleles. In addition, the causes of HL in some populations such as Baluchi are likely more homogenous than other parts of east Iran. This review highlights the importance of *GJB2* mutations in development of HL in eastern parts of Iran and are of great importance for successful disease management and interventions, mainly for genetic counseling and cochlear implants

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Narrow-band chirp and tone burst auditory brainstem response as an early indicator of synaptopathy in industrial workers exposed to occupational noise

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Summary **This study aims at characterizing and comparing the findings of auditory brainstem response (ABR) using narrow-band chirp (NB-chirp) and tone burst (TB) for both latency and amplitude parameters among those exposed to occupational noise and to determine which among the two serves as a better indicator of noise-induced cochlear neuropathy. Forty adult males in the age range of 20-35 years were considered, wherein 20 of them were exposed to noise > 80 dB (A) for 8 hours per day constituting Noise-exposed group; and Control group consisted of 20 individuals without occupational noise exposure. ABR was recorded using NB-chirp and TB for four frequencies at 80 dB nHL through Etymotic Research – 3A (ER-3A) Insert phones using Interacoustics Eclipse EP-25 in individuals with and without noise exposure. MANOVA was performed to compare between TB ABR and NB-chirp ABR between the two groups. Statistical analysis revealed a notable difference for NB-chirp comparisons between the two groups at three frequencies: 500 Hz, F(1, 38) = 10.6; 1000 Hz, F(1, 38) = 7.91; and 2000 Hz, F(1, 38) = 6.64. Whereas, the difference was evident at only 500 Hz: F(1, 38) = 4.98 in case of TB ABR. However, there was no significant difference seen at any of the frequencies for amplitude parameters in both TB and NB-chirp ABR. Latency of wave V using NB-chirp was considered to be a better indicator compared to TB, acting as a better clinical tool in early identification, diagnosis, and monitoring of noise induced hearing loss (NIHL).**

Keywords: Auditory brainstem response, narrow band-chirp, tone burst, noise induced hearing loss, cochlear synaptopathy

1. Introduction

We all experience sound in our environment through different sources which are at safe levels of hearing and do not cause any discomfort. But, there are sounds, which are loud enough to cause damage to the hearing structures and hence cause hearing loss depending on the exposure duration (*1*). The effects of noise-induced hearing loss (NIHL) include auditory and non-auditory effects. The two sets of auditory effects include the effects that are noticeable after a certain duration of exposure to noise and the effects that are seen during the course of noise exposure. The after effects of noise can lead to temporary or permanent hearing loss, which arises due to damage in peripheral or higher auditory centers.

Chronic exposure to noise in industrial workers that affect bilateral cochlea causes high-frequency sensori neural hearing loss (SNHL) with 4000 Hz notch (*2*). Further, they observed that around 39% of industrial workers who were exposed to noise levels > 87.3 dB (A), for 8-12 hours per day suffered from SNHL (*3*).

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Regular exposure of cochlear amplifiers to high-level noise may yield irreversible damage to them (*3*). An ideal test used for identifying the shifts observed in cochlear functioning would be otoacoustic emissions (OAEs). OAEs are preferred over pure tone audiometry for early identification of NIHL because, they are sensitive to minor damage and also can be monitored easily due to their objectivity and speed (*4*). However, in early stages, there may not be any evident threshold shifts even in the presence of underlying efferent system damage. There is evidence from animal as well as human studies which suggest that, even moderate exposure to acoustic stimulus, which can cause temporary threshold shift can destroy the connections between the auditory nerve fibers (ANFs) and cochlear hair cells causing synaptopathy (*5-7*). This type of damage to the synapse, which causes no permanent threshold elevation, is termed hidden hearing loss (*8*). It was reported that neural degeneration in ears with noise-induced threshold shifts in mice subjected to mild acoustic trauma suggested that normal hearing thresholds can be accompanied by impaired function of efferent fibers that project from the brainstem to the cochlea (*5*). Hence, assessment at the brainstem level provides valuable information on early identification of NIHL. There is also reduction seen in compound action potentials and spontaneous neural activity induced by noise exposure in electrophysiological tests (*9*).

Auditory brainstem response (ABR) is an electrophysiological test to measure the functional integrity of brainstem auditory structures (*10*). ABR is considered to be a valuable tool in evaluating auditory functioning, including difficult to test populations. The brainstem auditory evoked potential or short latency potential represents a series of neuro-electric potentials recorded from electrodes placed on the scalp. In order to assess different frequency regions within the cochlea, various stimuli have been employed in ABR measurements such as click, tone burst (TB) and speech stimuli. Out of these stimuli types, a brief tonal stimulus gives a better representation (*11*). Chirp stimuli are brief tonal stimuli designed to compensate for the delay in time for basilar membrane travelling wave in order to improve the temporal synchrony between the neural elements that usually are asynchronously activated by a brief stimulus such as a click (*12*). It is said that such compensation yields higher temporal synchronization of the neural structures that contribute to elicitate ABR, and also produces extremely large response amplitudes (*12*). A study done on individuals with and without noise exposure using click and Claus Elberling chirp stimuli (CE-chirp) indicated that there was a significant delay in latency as well as reduced amplitude in individuals exposed to noise. However, this was not seen when clicks were used. Hence, it was concluded that responses obtained with CE-chirp stimuli is an effective tool in identifying the early pathological changes caused due to occupational noise exposure when compared to click-evoked ABR (*13*). NB-chirps are constructed with an octave bandwidth wherein there is super position of four one-octave-wide chirps centered at 500 Hz, 1000 Hz, 2000 Hz, and 4000Hz which are capable of improving the neural synchronization and also provide frequency-specific information (*12,14*). A complete evaluation should contain frequency-specific information because it provides better detail about the configuration of the hearing loss. TB is a short-time signal consisting of a single tone, which is utilized for testing, measurement, and/or calibration. TB is a spectrally narrow stimulus. Studies have shown that as the frequency increases, the peak V latency decreases (*12*). The responses from these stimuli are also frequency specific with consideration that, with the use of frequency specific stimuli like TB and NB-chirps, the spectral splatter is reduced to some extent and reduces the participation of other regions of the cochlea. According to the literature, frequency specific TB can be a better predictor of pure tone thresholds than click evoked ABR (*15,16*). Also, since NB-chirp is constructed with an octave bandwidth centered at 500 Hz, 1000 Hz, 2000 Hz, and 4000Hz, it would also yield frequency-specific information (*12-14*).

The aim of the present study is to show the effectiveness of two different frequency specific stimuli available namely, NB-chirp and TB in ABR, and to show which of the two are ideal for ascertaining the auditory system changes that arise due to NIHL, with normal peripheral hearing sensitivity, in turn helping early identification of cochlear neuropathy resulting from NIHL. Two groups consisting of individuals with and without noise exposure were involved and the effect of two different stimuli, NB-chirp and TB on the auditory system were compared in this study. Between-group comparisons were made wherein, the same stimuli were compared across two groups, that is, TB of Control group was compared with TB of Noise-exposed group and the same for NB-chirp ABR.

2. Materials and Methods

2.1. *Participants*

Forty adult male participants were selected randomly from a single work place and were divided into two groups of twenty individuals each. "Control group" included individuals who were not exposed to occupational noise (age range = 20 to 35years, mean = 23.5 years) and the "Noise-exposed group" included individuals who were exposed to noise greater than 80 dB(A) [mean = 87.5 dB(A)] for a duration of 8 hours per day in their workplace (age range = 20 to 35years, mean = 27.75 years) for a minimum time period of 3 years (range = 3 to 5.6 years). The noise measurement

at the working place was performed using a calibrated SLM (B & K model 2270) with windshield for a duration of 5 minutes at each site. The microphone was placed at the ear level within a diameter of 1 meter. For measuring the amount of noise exposure, the tripod stand with the microphone was placed behind the individual's ear with approximately 180° azimuth within a distance of 1 meter. The subjects considered were non-smokers and non-alcoholics. None of them were under any medications for other ailments or using any type of hearing protective devices. Not all participants expressed difficulties during communication. However, the major communication disability reported by these individuals was difficulty in listening in the presence of background noise and difficulty in talking over the phone. Each subject gave written informed consent at the outset.

2.2. *Procedure*

As a first step, a detailed case history was taken from all the participants to rule out any pathological conditions of the auditory system and to procure information about their working environment, work experience and listening difficulties faced by them. All participants from control group and noise-exposed group were subjected to pure tone audiometry, immittance and ABR. Pure tone audiometry for octave frequencies between 250 to 8000 Hz were tested using a dual channel diagnostic audiometer (calibrated as per ANSI S3.6, 1996). Only those participants whose hearing sensitivity was < 20 dB HL at each frequency in the aforementioned frequency range (in both groups) without any otologic, psychological or neurological dysfunction were selected for the study. The 20 dB HL threshold criteria were fixed in order to rule out any peripheral hearing loss in the participants. The mean pure tone average at four frequencies (500Hz, 1000Hz, 2000Hz and 4000Hz) was 8.50 dB for control group and 9.25 dB for noise-exposed group participants. Speech recognition thresholds were obtained using Kannada paired words and Speech Identification Scores (SIS) using Phonetically Balanced (PB) word lists in Kannada language (*17*). The mean SIS scores were 7.25 for control group and 7.75 for the occupational noise exposed group. Immittance evaluation, which includes both tympanometry and acoustic reflexes was done to rule out any middle ear dysfunction.

The participants were also tested with distortion product otoacoustic emissions (DPOAEs) at 8-points per octave from 1000Hz to 8000Hz at 80 dB to assess the outer hair cell (OHC) functioning. They were recorded with a frequency ratio of 1.22 for the primary tones and the level of f2 primary was kept 10 dB less than f1 level. The ABR assessment was carried out in a sound treated room using the Interacoustics Eclipse EP-25 system. The electrical potentials were obtained with electrodes placed at Fz, M1, M2; and ground at Fpz position. The measured potentials were recorded with impedance below 5kΩ at all electrodes and the stimulus was presented through ER-3A insert phones. The assessment was done with two stimuli namely, narrowband-chirp (NB-chirp), and TB of 2-0-2 cycle at four different frequencies 500 Hz, 1000 Hz, 2000 Hz and 4000 Hz. The stimulus level was kept constant at 80 dB nHL at a repetition rate of 11.1/sec. A band pass filter of 100-3000 Hz was used and the data were collected in a 12 ms time window for NB-chirp and 14 ms for TB. One thousand five hundred sweeps were averaged at each presentation for two replications and the average was taken. The absolute amplitude and absolute peak latencies were recorded for peak V in all four frequencies between the groups. The peaks were marked by two experienced audiologists for reliable measures. The data analysis was done using SPSS, software version 21 for 40 ears. Shapiro-Wilk's test for normality was administered. The amplitude of DPOAEs followed a non-normal distribution and hence, a non-parametric test was administered. For ABR, the latency parameter was observed to be within normal distribution, and hence a parametric test was administered. Whereas, a non- parametric test was administered for amplitude parameters because the data did not follow normal distribution. $p < 0.05$ was used to verify the level of significance during statistical analysis.

3. Results

3.1. *Comparison of DPOAEs amplitude between Control group and Noise-exposed group*

Because the data followed non-normal distribution, Mann-Whitney *U* test was administered. DPOAEs were present for both groups wherein, the criteria of amplitude greater than 6 dB were considered for OAEs to be present. It was evident that the DPOAEs of the participants from both groups had no significant difference across most of the frequencies. There was a significant difference observed only at frequencies of 1001Hz with Z = -2.33, $p < 0.05$, and r′ = 0.03; 3369 Hz with Z = -2.08, $p < 0.05$, and r′ = 0.03; and 7336 Hz with Z = -2.09, $p < 0.05$, and r′ = 0.03. However, there was no trend observed in amplitude difference across frequencies between the two groups. The median amplitude of DPOAEs across frequencies between control group and noise-exposed group is presented in Figure 1.

3.2. *Comparison of the absolute latency and amplitude of peak V between Control group and Noise-exposed group for TB ABR*

Latency Comparisons: Descriptive statistics were

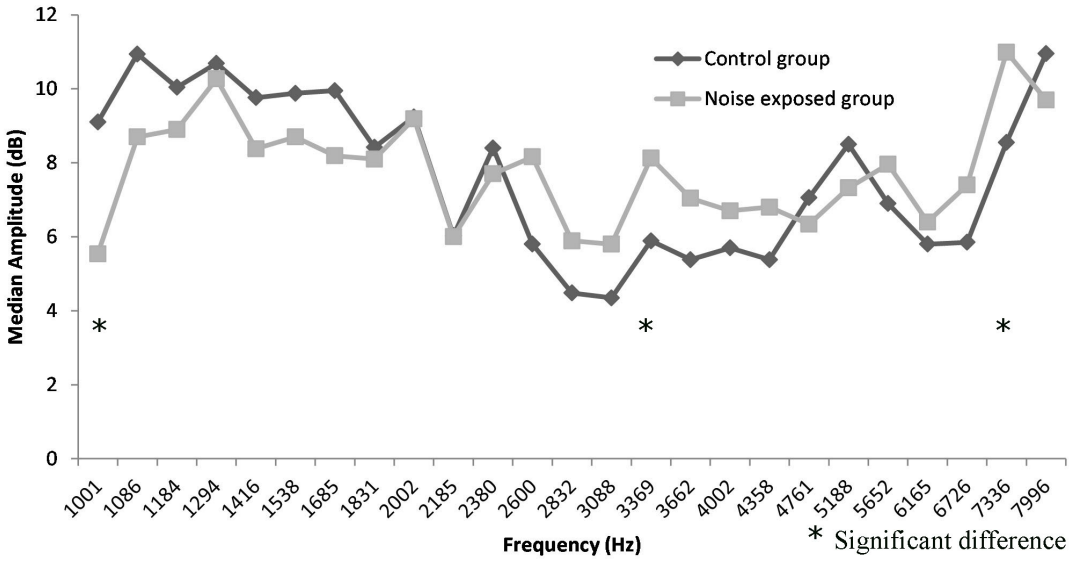

Figure 1. Median amplitude of DPOAEs in Control group and Noise-exposed group across different frequencies. DPOAEs, distortion product otoacoustic emissions.

carried out to find the mean and standard deviation of peak V between control group and noise-exposed group for TB. The mean latency for 500 Hz was found to be 7.92 ms (SD = 0.78) for Control group and 7.45 ms (SD = 0.53) for Noise-exposed group; at 1000 Hz the mean latency was 7.03 ms (SD = 0.65) for Control group and 6.88 ms (SD = 0.38) for Noise-exposed group; at 2000 Hz the mean latency was 6.36 ms (SD = 0.65) for Control group and 6.18 ms (SD = 0.24) for Noise-exposed group; and at 4000 Hz the mean latency was found to be 5.77 ms (SD = 0.22) for Control group and 5.85 ms (SD = 0.25) for Noise-exposed group. To compare the absolute latency of peak V for TB ABR between Control group and Noise-exposed group, MANOVA was administered. A statistically significant difference ($p < 0.05$) was exhibited at only 500 Hz, $F(1, 38) = 4.98$, $\eta^2_p = 0.17$. But, there was no statistically significant difference ($p > 0.05$) evident at 1000 Hz, $F(1, 38) = 0.78$, $\eta^2_p = 0.02$; 2000 Hz, $F(1, 38) = 1.28$, $\eta^2_p = 0.33$; and 4000 Hz, $F(1, 38) = 0.97$, $\eta^2_p = 0.03$.

Amplitude Comparisons: The median amplitude for TB ABR at 500 Hz was found to be 0.27 µV for both control group and noise-exposed group; at 1000 Hz the median amplitude was 0.19 µV for Control group and 0.25 µV for Noise-exposed group; at 2000 Hz the amplitude was 0.22 µV for Control group and 0.21 µV for Noise-exposed group; and at 4000 Hz the amplitude was found to be 0.2 µV for Control group and 0.19 µV for Noise-exposed group. Mann-Whitney test was carried out to compare the absolute amplitude of peak V for TB between the groups. There was no statistically significant difference ($p > 0.05$) observed at all four frequencies.

3.3. *Comparison of the absolute and the absolute latency of peak V between Control group and Noise-exposed group for NB-chirp ABR*

Latency Comparisons: Descriptive statistics was carried out to find the mean and standard deviation of peak V between control group and noise-exposed group for NB-chirp. The mean latency for 500 Hz was found to be 2.45 ms (SD = 0.68) for Control group and 3.13 ms (SD = 0.63) for Noise-exposed group; at 1000 Hz the mean latency was 3.49 ms (SD = 0.72) for Control group and 4.06 ms (SD = 0.53) for Noise-exposed group; at 2000 Hz the mean latency was 4.54 ms (SD = 0.59) for Control group and 4.99 ms (SD = 0.51) for Noise-exposed group; and at 4000 Hz the mean latency was found to be 5.38 ms (SD = 0.32) for Control group and 5.68 ms (SD = 0.62) for Noise-exposed group. To compare the absolute latency of peak V for NB ABR between Control group and Noise-exposed group, MANOVA was administered. A statistically significant difference was exhibited at 500 Hz, $F(1, 38) = 10.61$, $\eta^2_p = 0.21$; 1000 Hz, $F(1, 38) = 7.91$, $\eta^2_p = 0.17$; and 2000 Hz, $F(1, 38) = 6.64$, $\eta^2_p = 0.14$. However, there was no statistically significant difference at 4000 Hz, wherein, $F(1, 38) = 3.5$, $\eta^2_p = 0.08$.

Amplitude Comparisons: The median amplitude for NB-chirp at 500 Hz was found to be 0.12 µV for Control group and 0.19 µV for Noise-exposed group; at 1000 Hz the median amplitude was 0.07 µV for both Control group and Noise-exposed group; at 2000 Hz the amplitude was 0.05 µV for Control group and 0.07 µV for Noise-exposed group; and at 4000 Hz the amplitude was found to be 0.09 µV for Control group and 0.11 µV for Noise-exposed group. Mann-Whitney test was carried out to compare the absolute amplitude of peak V for NB-chirp between the groups. There was no statistically significant difference ($p > 0.05$) observed at all four frequencies.

The overall results indicate that, there was a significant difference observed for latency parameter at only 500Hz for TB ABR, whereas, the difference was

evident at 500Hz, 1000Hz and 2000Hz for NB-chirp ABR. However, there was no significant difference seen at any of the frequencies for amplitude parameter in both TB and NB-chirp ABR. The graphs representing the comparisons are shown in Figure 2 and Figure 3 for latency and amplitude parameters respectively.

4. Discussion

The study aimed to compare the absolute peak latency and absolute amplitude using TB and NB-chirp, across two groups: Control group (without occupational noise exposure) and Noise-exposed group (with occupational noise exposure). Results indicated characteristic differences in NB-chirp between the two groups in ABR recordings. Cochlear synaptopathy is a condition where there is no evident loss in the hair cells but an irreversible loss of synapses between the inner hair cells (IHCs) and the ANFs is seen (*5*). OHC dysfunctioning leads to a loss of sensitivity and a reduction in frequency selectivity. When assessed audiometrically in quiet, the thresholds are in normal limits as the OHCs are intact in spite of up to 80% of synaptic loss corresponding to IHC damage (*18*). Hence, the presence of DPOAEs in both the groups showing no significant difference in the amplitude indicates damage at the IHC or at the synaptic level. To assess the functioning of IHC/auditory nerve synapse ABR would be a better tool, and more particularly a frequency specific stimuli would tell us the functioning characteristics at different regions. For the TB stimulus, latency values decreased as frequency increased which followed the expected trend. Supported by several studies, which have shown that at lower frequencies, the latencies are prolonged due to responses that arise from the apical region of the cochlea (*19-22*). For NB-chirp stimulus, the ABR latency values decreased with decrease in frequency. This pattern was the opposite of that which occurred for the TB stimulus (*23,24*). This can be explained by the fact that, there are shorter response latencies in the NB-chirp ABR due to the temporal references [0 ms] (*24*). The arrival time at the eardrum is the 8000 Hz component, which compensates for frequency delay characteristics of the basilar membrane (*22*). The NB-chirp 500 Hz, 1000 Hz, 2000 Hz and 4000 Hz can be considered as a subset of the chirp evoked ABR, thus

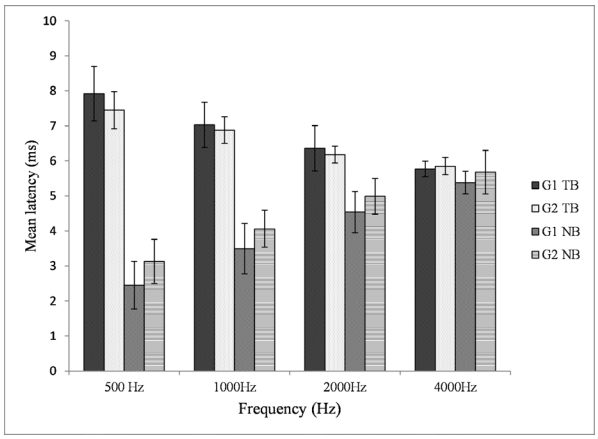

Figure 2. Mean latency (ms) of peak V for TB and NB-chirp ABR in Control group and Noise-exposed group across different frequencies. TB, tone burst; NB-chirp, narrow-band chirp; ABR, auditory brainstem response.

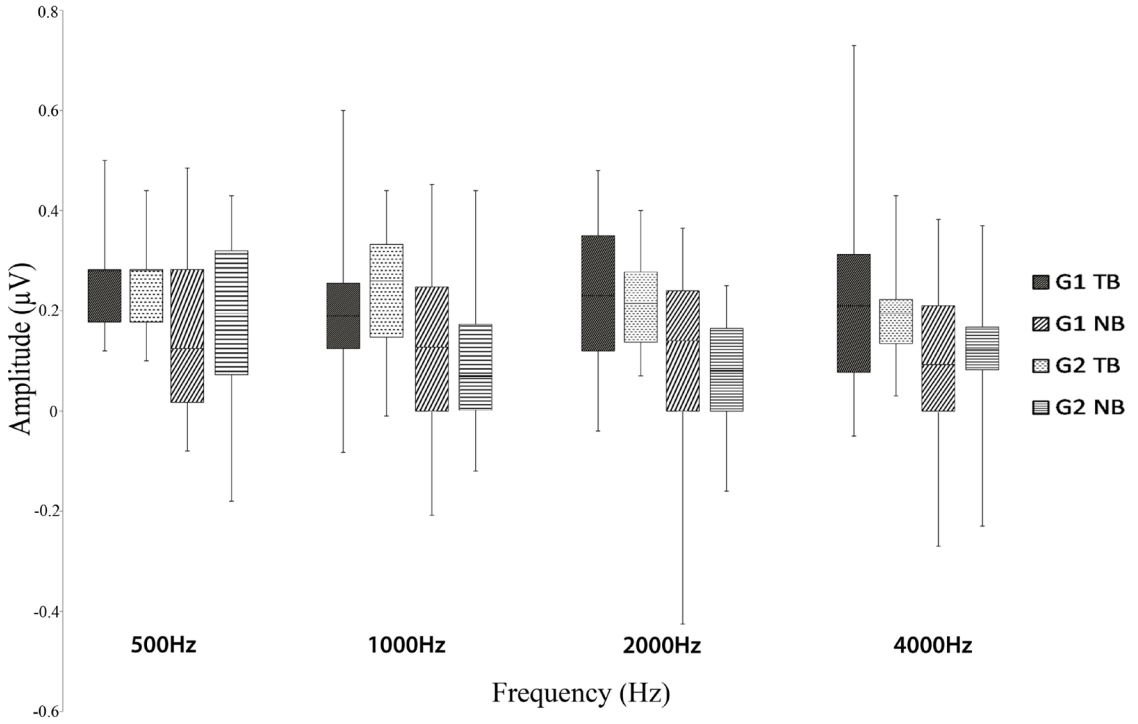

Figure 3. Amplitude (µV) of peak V for TB and NB-chirp ABR in Control group and Noise-exposed group across. TB, tone burst; NB-chirp, narrow-band chirp; ABR, auditory brainstem response.

exhibiting shorter latencies compared to TBs for low frequency stimuli. Also, because NB-chirp provides maximum stimulation to the cochlea, the delay in the cochlear travelling wave will be compensated by attaining different timing across each of the frequencies (*24,25*). That is, the regions centered at 500 Hz and 1000 Hz receives the stimulus earlier, giving shorter peak latency. This trend of latency change with frequency of the stimuli was consistently observed in both groups.

In the current study, there was a significant difference observed in terms of latency at 500Hz, 1000Hz, and 2000Hz for NB-chirp stimuli. But, there was no significant difference observed between the two groups at 4000Hz, although the latency was prolonged in the noise exposed group. A cochlear histopathological study conducted on mice by Sergeyanko, Lall, Liberman, & Kujawa in 2013 (*26*), revealed similar results *i.e*. IHC ribbon losses were initially greater in the apical region when compared to base, but with increasing age, the synaptopathy spread throughout the cochlear spiral, whereas, OHC ribbons were well preserved, which approximated to < 10% of loss across all frequencies. The reason for the discrepancy is unclear, but one possibility could be that the surviving hair cells in the region of the cochlea corresponding to very high frequencies *i.e.* 16-40 kHz may be present, but functioning would be abnormal (*27,28*) and thus, we hypothesize that this delay in latency values, which is more evident in lower frequencies could be due to abnormal functioning of the hair cells in the higher frequency regions which might hamper further signal conduction along the basilar membrane to the low frequency regions in individuals with occupational noise exposure.

The amplitude of the TB was more than the amplitude of the NB-chirp at all four tested frequencies. This is because the chirp evoked ABRs exhibit a non-monotonic level-dependent behavior, which is caused by the broadening of neural excitation as the level increases (*25*). At low intensity levels, each frequency corresponds to a narrow frequency region of the basilar membrane and hence, each component adds up in phase (*29*). At high levels, there will be a broader excitation on the basilar membrane, which results in desynchronization and causes the peak V amplitude to reduce (*23*). Also, the higher ABR amplitude for TB can be explained in terms of shaping of a pure tone with its additional sidebands due to its low frequency specificity. This effect can become evident in normal hearing subjects, especially at high stimulation levels. Some of the earlier studies, which compared ABR for the TB and the narrow band CE-chirp stimuli at various levels explained that, the narrow band CE chirps have greater amplitude than the TBs as a result of simultaneous depolarization at the specific frequency region of the cochlea accounting for the design of the narrow band CE-chirps (*24,25*). However, this pattern is not observed for higher stimulus levels [80 dB nHL], where the TBs may have relatively better amplitude [for 500 Hz], and/or there may not be a significant difference between the stimuli across the frequencies 1000 Hz, 2000 Hz, and 4000 Hz. The observed pattern was explained by the upward spread of the excitation at low levels where, each frequency component excites specific area in the cochlea. But, at higher levels the excitation is present for the broader area around the specific frequency region, which is represented as reduced amplitude (*24,25*). In line with these studies, there was no significant difference observed in the amplitude of peak V between the TB and the narrow band CE chirp stimuli at 80 dB nHL.

It is evident that although the participants from both groups had good pure tone thresholds, individuals with occupational noise exposure showed prolonged latencies in comparison with the normal population when NB-chirp stimuli was used and no difference in the amplitude parameter for NB-chirp, whereas, there was no significant difference observed for both latency as well as amplitude parameters in TB stimuli. Increased noise exposure results in reduced amplitude of the ANF-generated ABR wave-I (*30*), which is consistent with the effects of cochlear synaptopathy on ABR wave-I generated in animals. Even though ABR wave-I amplitude offers an objective measure of loss of ANFs in animals, it is difficult to measure robustly across different intensities in humans. However, wave-V of ABR, which is generated in the lateral lemniscus and inferior colliculus (*31*) is robustly represented in humans and can be recorded even at low levels of stimulation and in the presence of background noise. Unfortunately, ABR wave-V amplitude is not reduced by cochlear synaptopathy (*26*). This shows that latency would be a better measure than amplitude at supra-threshold (80 dB nHL) level stimulation for the early identification of cochlear synaptopathy. Kujawa and Liberman (2009) (*5*) studied the effects of noise exposure on mice, and their findings show that even if the thresholds had resumed to normal, with intact cochlear cells, there was some loss of afferent nerve terminals and delayed degeneration of the cochlear nerve. Thus, the significant difference seen can be evidence of the early neuro-physiological changes that are underlying even when the thresholds indicate normal hearing sensitivity. These changes may further increase and gradually lead to a greater effect on the auditory system (*5*). Hence, even when the thresholds are clinically normal, the prolonged ABR latency results obtained using NB-chirp stimuli at higher intensities helps in determining cochlear synaptopathy. The major limitation of the study was the number of participants in the study because it was limited to 30. To generalize the findings a larger sample size would have been appropriate.

5. Conclusion

The study evaluated changes in both amplitude and latency parameter of wave V of TB and NB-chirp ABR in individuals exposed to occupational noise. There was no change seen between the groups when the comparison was made in terms of amplitude parameters. The significant difference observed for latency parameters at 500Hz, 1000Hz and 2000Hz for NB-chirp ABR, whereas at only 500Hz for TB ABR suggests that NB-chirp ABR is a better clinical tool in identifying damage at a higher level of the auditory system. This difference can be evidence of the early neuro-physiological changes happening at the core level even when the thresholds indicate normal hearing sensitivity.

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Brief Report

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Syndromic progressive neurodegenerative disease of infancy caused by novel variants in HIBCH: Report of two cases in Colombia

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Summary **3-Hydroxyisobutyryl-coenzyme A (CoA) hydrolase deficiency (HIBCHD; MIM: #250620) is a rare autosomal recessive inborn error of metabolism caused by a defect in the HIBCH enzyme, resulting in a deficiency of the conversion of 3-hydroxy-isobutyryl-CoA to 3-hydroxy-isobutyric acid, a critical step in valine catabolism. This neurodegenerative disease of infancy is associated with hypotonia, developmental delay, cerebral atrophy and lesions in the basal ganglia on magnetic resonance imaging (MRI). In this study, we describe two unrelated patients with infantile-onset progressive neurodegenerative disease and mutations in HIBCH identified using whole exome sequencing (WES). In Case 1, WES revealed a novel homozygous variant in the *HIBCH* gene: c.808A>G (p.Ser270Gly). In Case 2, a novel compound heterozygous mutation in the *HIBCH* gene is described: c.808A>G (p.Ser270Gly) and c.173A>G (p. Asn58Ser). Parent analysis revealed that c.808A>G (p.Ser270Gly) was inherited from the father and c.173A>G (p. Asn58Ser) from the mother. These novel mutations were predicted as a disease-causing mutation. Plasma acylcarnitine analysis was normal in both patients. Physical examination showed similar features, such as axial hypotonia and spastic hypertonia in the legs. The first patient presented with difficult-to-treat seizures, while the second patient has not yet experienced documented seizures. In conclusion, our findings would widen the mutation spectrum of HIBCH deficiency and the phenotypic spectrum of the disease. The potential genotype–phenotype correlation would be profitable for the correct diagnosis, treatment and integral management of patients with HIBCH deficiency.**

Keywords: 3-hydroxyisobutyryl-CoA hydrolase deficiency, amino acid metabolism, inborn errors, inborn errors of metabolisms, seizures, hereditary neurodegenerative diseases

1. Introduction

In humans, essential amino acids, such as leucine, isoleucine and valine, contribute to energy production through catabolism (*1*). When parts of this catabolism are interrupted, heterogeneous groups of diseases are generated, normally called inborn errors of metabolism. The term usually refers to primary disorders affecting the metabolism of amino acids, organic acids, lipids and complex carbohydrates that interfere with healthy brain development leading to developmental delay or intellectual disability (*2*).

3-Hydroxyisobutyryl-coenzyme A (CoA) hydrolase

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deficiency (HIBCHD; OMIM #250620) is a rare inborn error of metabolism caused by a defect in the HIBCH enzyme, resulting in deficiency in conversion of 3-hydroxy-isobutyryl-CoA to 3-hydroxy-isobutyric acid, a critical step in valine catabolism (*3*). This deficiency presumably leads to accumulation of toxic metabolites in mitochondria (*3*). Only 16 patients from 10 unrelated families, 6 of which are consanguineous, have been reported in the worldwide literature (Supplementary Table S1, *http://www.irdrjournal.com/action/getSupplementalData.php?ID=44*). This autosomal recessive condition is characterised by the developmental delay of motor milestones in early infancy, which is associated with episodes of ketoacidosis and high concentrations of pyruvate and lactate in the cerebrospinal fluid (*4*) and neurologic regression within the first year of life. Magnetic resonance imaging (MRI) abnormalities are striking for bilateral involvement of the basal ganglia with varying degrees of white matter atrophy (*2*).

We report on two unrelated Colombian patients with neurologic disease. Whole exome sequencing (WES) was performed in both cases and two novel mutations in the *HIBCH* gene were identified. Patients presented with remarkable psychomotor developmental delay and in one case infantile neurodegenerative disease. The suspicion of an inborn error of metabolism existed in both cases, but difficulty in diagnosis was due to heterogeneity of the clinical presentation.

2. Patients and Methods

Clinical and family history and diagnostic findings were obtained in a clinical setting. The following clinical variables were obtained for each proband: evidence of consanguinity, abnormal perinatal period, anthropometric variables, age of diagnoses, developmental milestones, symptoms and signs, dysmorphic facial features, brain image abnormalities, blood and biochemical analysis, muscle skeletal biopsy results, status of variant inheritance and survival rate. Written informed consent to participate in the study and to publish clinical information and photographs was obtained from the parents. A local ethics committee of the Faculty of Health Sciences of the Universidad Icesi approved the study.

2.1. *Case 1*

A 1-year-old Colombian girl, born to consanguineous parents, was the product of a first pregnancy that ended *via* caesarean section at 39 weeks due to a prolonged delivery stage. She weighted 2,725g (between 9th and 25th percentile), seize 50cm (9th and 2nd percentile) and head circumference 35.5cm (91st and 75th percentile) according to world health organization growth chart. There was no family history of any heritable

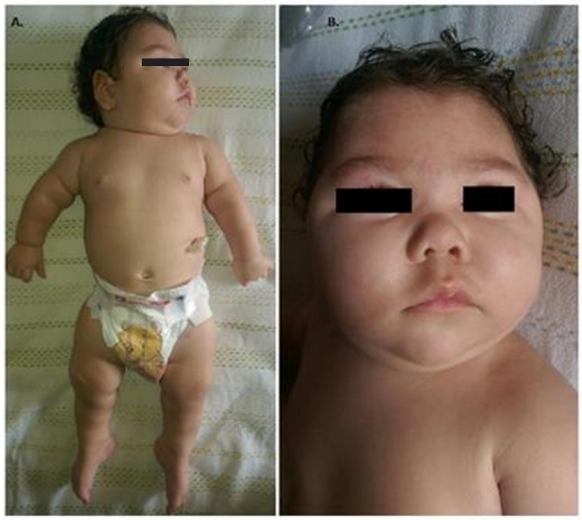

Figure 1. Case 1 phenotype. (A), Front profile at age 1 year shows short forehead, wide palpebral fissures, epicanthus fold, synophrys, nasal bone hypoplasia, low nasal bridge, bulbous nose, prominent philtrum groove, small mouth with cupid's bow, and microcephaly; **(B)**, Spastic legs show generalised hypotonia. Permission was obtained from the patient's parents for presentation.

progressive neurodegenerative disease. The patient was frequently hospitalised for persistent vomiting, anorexia, irritability, swallowing difficulties, poor feeding, psychomotor developmental delay, no language skills and developmental regression since she was 2 months old. For that reason, a gastrostomy tube was placed and Nyssen fundoplication was performed to treat gastroesophageal reflux disease (GERD) at 3 months old. At 9 months old, multiple episodes of seizures and myoclonus developed. Physical examination showed a short forehead, wide palpebral fissures, epicanthal fold, synophrys, nasal bone hypoplasia, low nasal bridge, prominent philtrum groove, small mouth with cupid's bow, microcephaly, punctate anterior fontanelle, axial hypotonia, decreased tendon reflex, spastic legs (Figure 1 A and 1B) and hepatomegaly, with severe milestones growth development delay. She has never achieved cephalic support, ability to sit, ability to walk or ability to speak. Multiple tests revealed an elevated pyruvate/lactate ratio and lactate in the blood and cerebrospinal fluid. Brain MRI showed bilateral hyperintensity in the basal ganglia in DWI sequences (Figure 2A) and hypodensity in ADC sequences (Figure 2C) due to restriction. A metabolic abnormality was suspected due to a vast number of differential diagnoses. Magnetic spectroscopy revealed elevations in lactate (Figure 2C and 2D), which were more pronounced in regions where abnormalities were seen (Figure 2A, 2B and 2F). T1 sequences (Figure 1E and 1F) showed hypointensity on basal ganglia, which enhance with contrast. Axial potency in T2, showed bilateral damage of the basal ganglia and general cerebral volume loss in white and grey matter, with a significant increase in the number of gyri and sulci (Figure 2G) and deeply generalized

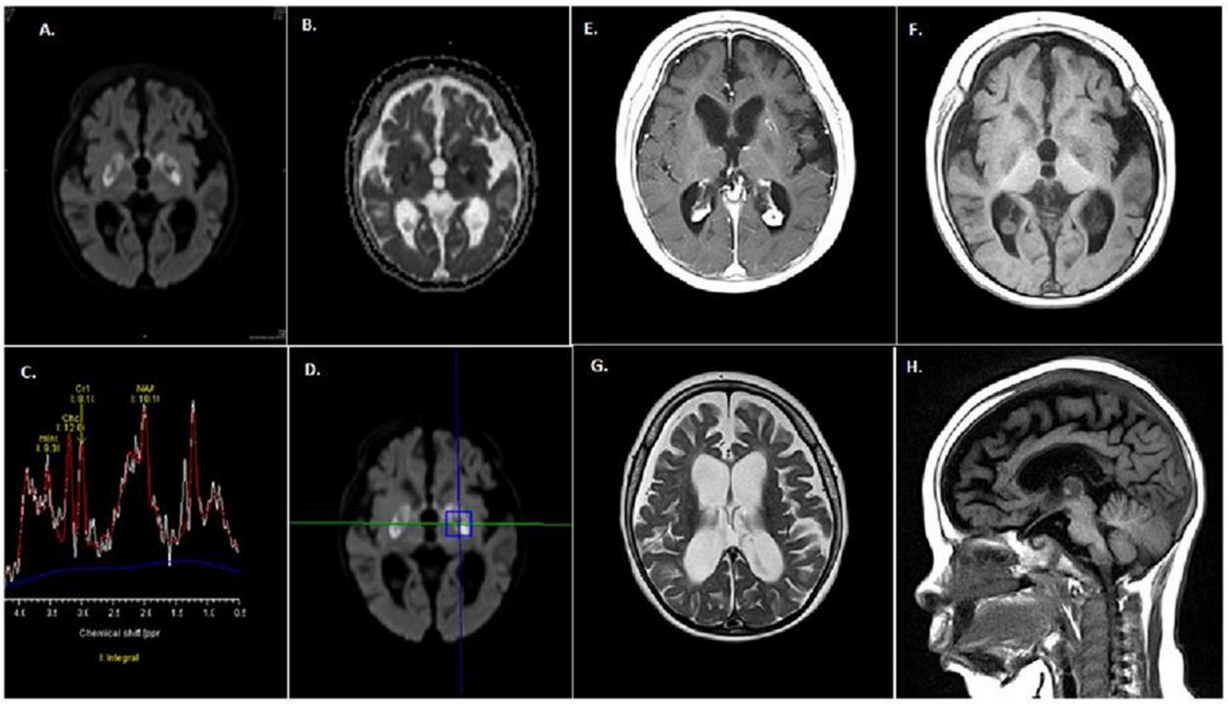

Figure 2. Case one MRI Images. (A), Axial section diffusion-weighted imaging (DWI) shows hyperintensity in the basal ganglia; **(B)**, Axial section shows apparent diffusion coefficient (ADC) basal ganglia restriction; **(C,D)**, Spectral curve with echo time (TE) of 30 mseg localises in the left globus pallidus; , where lactate and lipids peak with high concentration of myo-inositol are observed; **(E)**, Axial T1 Gadolinium (Gad), shows enhancement the basal ganglia **(F)**, Axial T1 image shows hypointensity in the basal ganglia; **(G)**, Axial T2, decline in brain volume. Gyrus, sulcus, subarachnoid space and ventricular system were prominent; (H), Sagital T1, section shows severe slimming of the corpus callosum and brain stem.

reductions in brain volume with predominance in grey and white matter, basal ganglia and pedunculus cerebri (Figure 2E). There was strong progression of cerebral atrophy compared to the other MRI scan and thickening of the corpus callosum and cerebral volume loss mainly in the supratentorial area (Figure 2H). Cardiac ultrasonography showed septal ventricular hypertrabeculation.

Her latest evaluation by the genetic consultant showed that the patient was still unable to walk, sit or communicate and a gastrostomy tube was kept due to increasing feeding difficulties. She received motor therapy and achieved mild cephalic support in prone position and slight gain of movement. Currently, she is receiving total enteral valine-free amino acid feeding, which allows changes in her behaviour including; less irritable but it does not contribute to control of seizure episodes and global development delay. Enzymes and the pyruvate dehydrogenase (PDHc) complex levels were not determined.

2.2. *Case 2*

A 4-year-old male from the southwest region of Colombia was the product of a 29-year-old mother and 43-year-old father (nonconsanguineous parents). The pregnancy was uncomplicated, and ultrasonography and prenatal care were normal. Vaginal delivery at week 36 of gestation was without complications. Birth weight was not documented and the cephalic perimeter at birth was 36 cm (54th centile). Apgar scores were 9 and 10 at 1 and 5 minutes, respectively. No phenotypic abnormalities or dysmorphism features were noticed at birth. Family history was positive for epilepsy in two cousins, and his mother had two previous miscarriages.

Generalised muscular hypotonia and poor weight increase were noticed 3 months after birth. Additionally, psychomotor development was significantly delayed. Neuropediatric follow-up at 6 months, due to previous clinical signs and symptoms, and brain MRI at 10 months revealed bilateral symmetrical hyperintense lesions in the basal ganglia on DWI sequences with hypointensity for restriction in the basal ganglia (Figure 3A and 3B) and fluid-attenuated inversion recovery (FLAIR) images globus pallidus (Figure 3D and 3E) associated with enlarged ventricles, suggestive of cerebral atrophy (Figure 3G and 3H). There were no structural brain anomalies, myelination defects or heterotopia. At 15 months of life, brain MRI spectroscopy demonstrated normal N-acetylaspartate, choline, creatinine and lactate peaks in the basal ganglia (Figure 3C and D), but imaging showed a compromise of the basal ganglia and posterior periventricular white matter (peritrigonal) without ischemia or active demyelination (Figure 3E and 3F). Axial potency T2, showed volume loss and hyperintensity on the basal

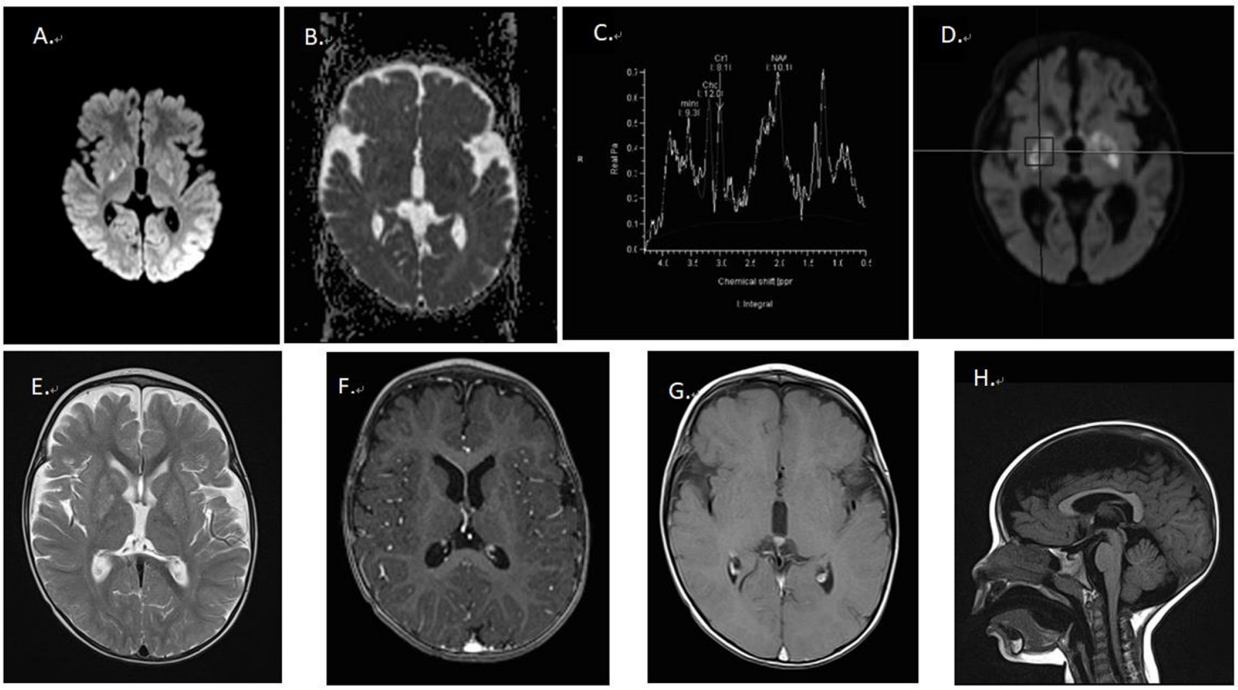

Figure 3. Case two MRI Images. (A), DWI-weighted imaging shows hyperintensity in the right basal ganglia; **(B)**, Axial section shows apparent diffusion coefficient (ADC) basal ganglia restriction; **(C,D)** Spectral curve with echo time (TE) of 30 mseg localises in the left globus pallidus; , where lactate and lipids peak with high concentration of myo-inositol are observed **(E)**, Axial T2 image, shows hyperintensity in the basal ganglia and decline in brain volume; **(F,G)**, Axial T1-weighted with and without contracts image shows hypointensity in the basal ganglia without enhanment ; **(H)**, Sagittal section shows slimming of the corpus callosum and brain stem.

ganglia (Figure 3G). Electroencephalogram (EEG) at 18 months showed background slowing activity and immature rhythms not expected at that age, and visual evoked potentials showed bilateral optic disorders with a right predominance. For that reason, Ophthalmology considered strabismus and torsional (rotary) nystagmus that required surgical correction. Auditory evoked potentials were normal.

Biochemical test results at 2 years of age were normal, including lactate, ammonium and liver function tests. Routine metabolic screening for aminoacidopathies and organic acidurias were normal. Plasma acylcarnitine analyses revealed no abnormalities. However, this screening did not routinely include hydroxy-C4-carnitine. At 3 years of age, he presented with an episode of gastrointestinal disturbance and fever that rapidly progressed to lethargy and worsened hypotonia. Similar clinical manifestations were present at 4 years of age, when he was hospitalised for upper respiratory symptoms with a fever that progressed to encephalopathy.

At age 4 years, he was medically assessed by Genetics. Physical examination revealed weight 15 kg (fourth centile), height 100 cm (first centile) and head circumference 51 cm (35th centile). A broad forehead, rotary nystagmus, convergent strabismus, facial symmetry with hypotonia, protruding tongue, ogival palate, axial hypotonia, spastic hypertonia predominantly in the lower limbs, hyperreflexia in the upper and lower limbs with bilateral extensor plantar reflex and hamstring contractures and immature clamp also were noted. The patient had insufficient trunk stability and was not able to sit or stand independently, or had he acquired any language. Repeat plasma acylcarnitine analysis was normal. The patient received an oral diet without difficulties and video fluoroscopic swallowing exam did not show any evidence of bronchoaspiration. At the moment he is taking multiple developmental therapies without any significant improvements.

Due to a large number of differential diagnoses, a blood sample for the patient and parents was collected, as well as medical records including clinical findings and the most relevant family history and WES was performed by using a trio approach with massive sequencing platform with Ion ProtonTM technology. The library preparation was designed with Ion AmpliSeq Exome technology (Life Technologies, Carlsbad, CA, USA) which captures > 97% of consensus coding sequences (CCDS; > 19,000 genes and > 198,000 exons) and flanking intronic regions (± 20 base pairs [bp]). Only variants in the coding and flanking intronic regions with a minor allele frequency (MAF) < 1.5% were evaluated. MAFs were based on the following databases: 1000 Genomes, dbSNP, Exome Variant Server (ESVor In-house), and Exome Aggregation Consortium (ExAc). In this study, we identified two novel mutations in a compound heterozygous state in

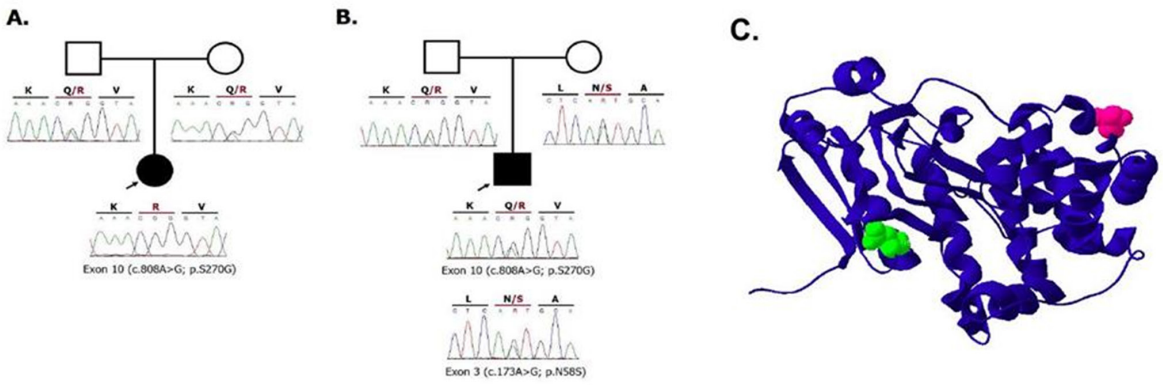

Figure 4. Pedigree information of patients and Sanger sequencing electropherogram of patients and parents. (A), Homozygous missense mutation (c.808A>G, p.Ser270Gly), both parents are carriers of the same mutation; **(B)**, Heterozygous compound mutation (c.808A>G, NM_014362.3) and (c.173A>G, p.Asn58Ser) at the cytoband 2q32.2 identified in patient. The first c.808A>G (p.Ser270Gly) was of paternal origin and c.173A>G (p.Asn58Ser) was of maternal origin. Variants were confirmed using Sanger sequencing. Both mutations are reported for the first time in this study to our knowledge; **(C)**, Visualisation of HIBCH *via* Swiss-PDB Viewer (v4.1.0) software. The locations of the mutations in the protein are shown. The amino acid marked *pink* corresponds to mutation c.173A>G, (p.Asn58Ser) and *green* to mutation c.808A>G (p.Ser270Gly) described in this report. Both are located in principal chain (aminoacids 33 to 386).

the *HIBCH* gene, the first c.808A>G (p.Ser270Gly) of paternal origin and c.173A>G (p.Asn58Ser) of maternal origin (Figure 4B and 4C).

To our knowledge, both variants have not been reported previously in the literature and the finding was confirmed using Sanger sequencing (Figure 4B) and was compatible with the diagnosis of 3-hydroxybutyryl-CoA hydrolase deficiency. Variant, functional prediction software tools SIFT and FATHMM classified the first variant (paternal origin) as Tolerated, while Mutation taster classified it as Disease-causing and Polyphen as possibly damaging (Score 0.613); the second variant (maternal origin) was predicted by SIFT, FATHMM, Mutation taster and Polyphen as Damaging (Disease-causing). No additional variants were identified.

3. Results and Discussion

HIBCHD is a rare inborn metabolism syndrome; in many cases, it can generate a secondary mitochondrial disorder caused by homozygous or compound heterozygous mutation in the HIBCH gene on chromosome 2q32 (*5*). The estimated incidence of HIBCH deficiency in the general population ranges from 1 in 127,939 in East Asians to 1 in 551,545 in Europeans (*6*), while there is no data as yet for the incidence in South America.

The prevalence of this disease is underestimated due to the nonspecific clinical presentation and its similarities with Leigh syndrome, which is a common neurometabolic disorder associated with different genes (*6*).

We reported two novel mutations in the *HIBCH* gene. The first, c.808A>G (p.Ser270Gly), was found in a homozygous state Case 1 by WES. To our knowledge, this mutation has not been previously reported as disease causing for *HIBCH* deficiency. Moreover, a phenotype correlation was found between the patient and the described phenotype by others in the medical literature (*1,7*), including cardiac defects (*7*). Additionally, parents carrying the same mutation and consanguinity provided evidence of autosomal recessive inheritance, previously described by others (*8*). Several *in silico* tools predict this mutation as damaging, based on the conservation of the position through the species. The second mutation, c.173A>G (p.Asn58Ser), was found to be of maternal origin in Case 2. Due to its location in the gene (Figure 4C), it was predicted by *in silico* tools as Disease-causing and called attention to the presence of the same mutation in two unrelated patients in our country.

HIBCH is a biallelic enzyme and its perturbation produces variable biochemical deficits in skeletal muscle, and a variable accumulation of toxic valine metabolites, the majority being methacrylyl-CoA (*9*). The high levels of this metabolite in multiple tissues cause multiple characteristics of this disease. Indeed, it is hypothesised that intramitochondrial methacrylyl-CoA reacts with thiol groups (cysteine and cysteamine conjugates) that are known to accumulate in multiple tissues particularly, liver, kidney and brain (*7*). Therefore, early and consequent dietary restriction might help to prevent neurodegeneration in HIBCHD (*9*).

We reported hepatomegaly and extensive brain damage in our patients and this metabolite produces glutathione and pools of cysteine depletion, a deficiency that could lead to oxidative damage (*3*). Furthermore, Methacrylyl-CoA could react with multiple essential residues containing cysteine, including pyruvate dehydrogenase complex (PDHc) and respiratory chain enzymes and reduce their activity (*10*), producing irreversible binding cofactors, such as CoA and lipoid

acid (*11*). A lack of CoA would be expected to inhibit the Krebs cycle and to decrease adenosine triphosphate (ATP) production. These effects are likely to vary according to the levels of oxidative stress. As a result, cell damage will be generated and abnormal MRI findings in the basal ganglia may be produced (Figure 2).

These findings can be illustrated in our patient by the high levels of multiple biochemical products measured in blood and urine, such as repeatedly elevated blood lactate levels, and a high ratio of lactate/pyruvate in the blood and in the cerebral fluid. On MRI, basal ganglia necrosis generated hyperintense lesions in the globus pallidus, cerebral atrophy, corpus callosum thinning and the steady decline of brain volume with slimming of the brain stem and pedunculus cerebri and high signal abnormality in these areas, without alteration of myelination. Similar findings have been detected on MRI in Leigh syndrome (*12,13*) as described previously (*8,14*) representing metabolic damage to the metabolites of valine catabolism accumulation.

4. Conclusion

We present a summary of the sociodemographic, clinical presentation, brain imaging abnormalities and mutations present in our cases and in patients previously described in the literature (Supplementary Table S1, *http://www.irdrjournal.com/action/getSupplementalData.php?ID=44*) to delineate the clinical spectrum of HIBCHD. Recognised signs include seizures, developmental delay, motor disorders, absence of milestones growth achievements and multiple brain abnormalities. Recognizable symptoms and signs will help clinicians to diagnose low prevalence inborn errors of metabolisms, such as HIBCH deficiency. Additionally, a novel mutation was identified in two unrelated cases in a different inheritance status. In the first case as homozygous status and in the second case as heterozygous status is added to the literature by the first cases published in south-America and Colombia with different clinical findings and variable phenotype expression.

To successfully evaluate a patient with delayed development, hypotonia, early feeding problems and deterioration of neurologic function, an inborn error of metabolism must be suspected, and MRI must be performed. As seen in our HIBCHD patients, suggestive lesions detected on MRI are generalised lack of white matter, global atrophy or hyperintense basal ganglia.

Different treatment options have been suggested for patients with HIBCH deficiency based on the physiopathology of the disease. Replacement of the primary energy source in patients to carbohydrates (*8*) and a low-valine diet with carnitine supplementation could reduce the production of methacrylyl-CoA in neuronal cells (*15*). Recently, antioxidants have been described as additional treatment. However, although they were effective in reversing basal ganglia abnormalities, they cannot prevent progression of the disease (*4*).

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Ethics

Written informed consent was obtained from the patient's parents for the publication of the case details and accompanying images. Data was collected following the Declaration of Helsinki Good Clinical Guidelines. This study was approved by the Ethics Committee of Fundación Valle del Lili.

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Brief Report

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Molecular spectrum and allelic frequency of different subtypes (1, 2, 3, 6 and 7) of Spinocerebellar ataxia in the Indian population

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Summary **Spinocerebellar ataxia (SCA) is a rare, heterogeneous genetic group of disorders with overlapping clinical features that arises as a result of the degeneration of Purkinje cells. The most prominent clinical feature of SCA is difficulty with whole body movements. The aim of the current study was to analyze the allelic frequency of normal repeat sizes in different SCA subtypes in the north Indian population. Blood samples were collected from 200 subjects, DNA was extracted, and then multiplex PCR and fragment analysis were performed using the ABI-310 genetic analyzer. The prevalent cytosine-adenine-guanine (CAG) repeat size or allelic frequency for SCA1, 2, 3 , 6, and 7 were 29 repeats (59%), 21 repeats (72.5%), 23 repeats (13.1%), 9 repeats (30%), and 3 repeats (75%), respectively. Results indicated that the normal repeats are shifting to lower or upper ranges in the Indian scenario, and similar findings have been reported in other previous studies. Thus, this and other studies have suggested that the normal range of repeats for various SCA in the Indian scenario needs to be redefined and should be confirmed by studies with larger samples and by functional studies.**

Keywords: Spinocerebellar ataxia, ataxia, triplet repeat disorder, repeats

1. Introduction

Spinocerebellar ataxia (SCA) is a slowly progressive, autosomal dominant disorder that is characterized by a marked intra-familial and inter-familial clinical variability. The global prevalence of SCA is 1 to 5 per 100,000 populations. There are several subtypes of SCA reported; the subtypes prevalent in India include SCA 1, 2, 3, 6, and 7. Studies have reported that SCA subtypes are primarily caused by triplet repeat expansion and in some cases by point mutations, deletions, and missense mutations.

Genes related to SCA subtypes (1, 2, 3, 6, and 7) are *ATXN1*, *ATXN2*, *ATXN3*, *CACNA1A*, and *ATXN7*, respectively. SCA is a disorder involving a triplet cytosine-adenine-guanine (CAG) repeat; the normal range of repeats for subtypes 1, 2, 3, 6, and 7 is 6 to 36, 15 to 31, 12 to 40, 4 to 18, and 4 to 19, respectively (according to the American College of Medical Genetics and Genomics, or ACMG). This disorder shows a phenomenon of genetic anticipation in which affected individuals in succeeding generations have an earlier age of onset and more severe clinical features in the next generation, due to the expansion of the repeat number during gametogenesis.

Several studies have reported that the normal and disease range of repeats for different triplet repeat disorders vary considerably between populations (*1-3*). Screening populations for the polymorphic range of repeats helps to establish the normal range of repeats for a particular geographical region, enabling proper molecular diagnosis. Moreover, repeats that are large but still within the normal range, referred to as large normal alleles, are known to be indicators of disease prevalence (*4-7*).

Several studies have sought to distinguish the normal range of repeats in different subtypes of SCA in different Indian populations (*8,9*). To explain the frequency of normal and numerous repeats and the prevalence of ataxia in a given population, the current study examined the five most common types of ataxia, namely, SCA1, SCA2, SCA3, SCA6, and SCA7, in samples from 200 healthy controls.

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Table 1. The primers used in Multiplex PCR for SCA subtypes 1, 2, 3, 6, and 7

Name	Fluorochrome	Sequence (5'-3')	Amplicon (bp)
SCA1 F	FAM	gcggtcccaaaagggtcagt AAC TGG AAA TGT GGA CGT AC	124+CAG
SCA1 R		ggtcccaaaagggtcagtCAA CAT GGG CAG TCT GAG	
SCA2 F	PET	aaaagggtcagt GGG CCC CTC ACC ATG TCG	59+CAG
SCA2 R		caaaagggtcagtCGG GCT TGC GGA CAT TGG	
SCA3 F	VIC	gcggtcccaaaagggtcagt CCA GTG ACT TTG ATT CG	161+CAG
SCA3 R		gcggtcccaaaagggtcagt TGG CCT TTC ACA TGG ATG TGA A	
SCA6 F	NED	caaaagggtcagt CAG GTG TCC TAT TCC CCT GTG ATC C	102+CAG
SCA6 R		aaagggtcagtTGG GTA CCT CCG AGG GCC GCT GGT G	
SCA7 F	FAM	gcggtcccaaaagggtcagtTGT TAC ATT GTA GGA GCG GAA	277+CAG
SCA7 R		gtcccaaaagggtcagtCAC GAC TGT CCC AGC ATC ACT T	

2. Materials and Methods

Blood samples were collected from 200 healthy subjects. Two 2 mL of peripheral venous blood was placed in a EDTA tube for DNA isolation using the standard phenol chloroform method. The quality and quantity of DNA was assured using agarose gel electrophoresis and spectrophotometry, respectively.

Multiplex PCR was performed to amplify SCA genes (Subtypes 1, 2, 3, 6, and 7) using chimeric primers (Table 1). A multiplex PCR reaction of 25 ul consisted of *ATXN1*, *ATXN2*, *ATXN3*, *CACNA1A*, and *ATXN7* gene primers (1.5 pmol each), 2 ul of dNTPs, 1.5 X buffer#1, Taqpolymerase (Thermo Scientific Dynazyme (2 U), 7.5ul Q-Solution, and 50-100 ng of genomic DNA purified from peripheral blood as described above.

Reactions were performed on the ABI 9700 thermal cycler for 1 cycle at 98°C for 5 minutes, 35 cycles at 98°C for 30 seconds, 58°C for 30 seconds, 72°C for 1 minute, and a final extension at 72°C for 10 minutes. After PCR, 1.5 uL of the PCR product was added to 4 ul of formamide (HiDyeformamide, Applied Biosystems Inc.) and 0.5 ul of GS500-LIZ. The solution was mix thoroughly and then denatured at 95°C for 5 minutes. Samples were injected into an ABI PRISM 310 genetic analyzer (Applied Biosystems Inc.) with a 47 cm long and 50 micrometer diameter capillary containing Performance Optimized Polymer-4 (POP-4, Applied Biosystems Inc.) for 5 seconds with an injection kV of 15.0, and samples were electrophoresed at 15 kV for 40 minutes at 65°C. Amplicon length was calculated in comparison to the GS500-LIZ molecular weight standard using the program Genescan (Applied Biosystems Inc.)

The size of PCR products was calculated automatically on the basis of a standard curve based on the internal size standard. Each allele represented the number of CAG repeats. While different individuals had the same alleles, these differed slightly in size (bp) from the theoretical values for the amplicon length of the trinucleotide-repeat region. To reliably define the alleles, those individuals with alleles whose sizes were close to the theoretical values were grouped together.

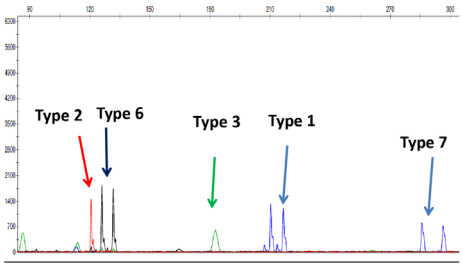

Figure 1. Representative result of multiplex PCR for five subtype of SCA.

Those values are used in the program Genemapper to determine the alleles of each loci.

3. Results and Discussion

This study used multiplex PCR to screen samples from 200 subjects. A representative multiplex PCR result is shown in Figure 1. Samples were used to determine the repeat sizes for the different SCA subtypes (1, 2, 3, 6, and 7) by calculating the amplicon sizes obtained from multiplex PCR.

The allelic frequency of the SCA subtypes 1, 2, 3, 6, and 7 was determined in the samples. The most prevalent CAG repeat size or allelic frequency was determined for the five SCA subtypes. For SCA1, the frequency was 59% (29 repeats), which means that 59% of the 200 samples had 29 repeats. This repeat size is the most common in the north Indian population. The allelic frequency of SCA subtypes 2, 3, 6, and 7 was 72.5% (21 repeats), 13.1% (23 repeats), 30% (9 repeats), and 75% (3 repeat). Allelic frequencies are indicated in Figure 2.

This study revealed the normal range of repeats for different subtypes of SCA in the Indian population. Based on the normal range of repeats in the 200 samples, the normal range of repeats are shifting to higher or lower ranges in some subtypes of SCA. This range varies in comparison to the normal range of repeats according to the ACMG. The range of repeats is shifting in the Indian scenario. Previous studies reported the range of repeats in the Indian population

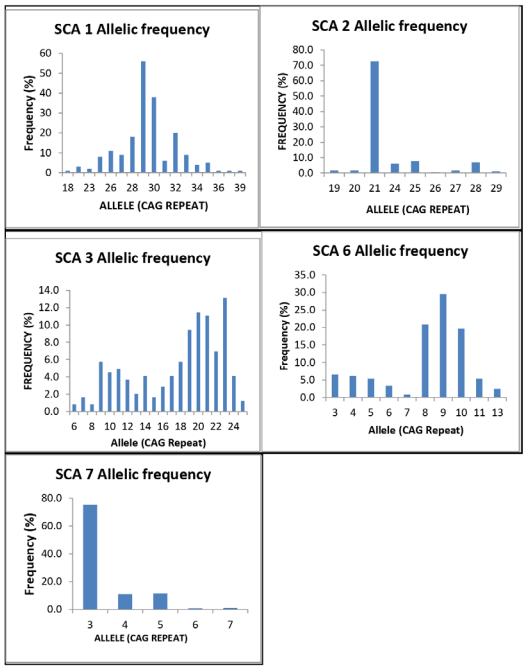

Figure 2. Allelic frequency of different subtypes (1, 2, 3, 6, and 7) of SCAs/.

and questioned whether those ranges needed to be redefined. Thus, studies with a larger sample size and functional studies need to be conducted to redefine the normal range of repeats in the Indian population.

SCA is an autosomal-dominant, adult-onset genetic disorder. It has multiple subtypes as have been reported, but i some subtypes are more prevalent n Indians such as subtypes 1, 2, 3, 6, and 7. This disease is caused by triple repeat expansion where the number of repeats exceeds the normal range.

The principal finding of this paper is a summary of the most common repeat sizes in different subtypes of SCA (1, 2, 3, 6, and 7) in the north Indian population. Different studies have reported different ranges of repeats for particular subtypes of SCA worldwide. In the current study, the most frequent number of repeats for SCA genes *ATXN1*, *ATXN2*, *ATXN3*, *CACNA1A*, and *ATXN7* was 29, 21, 23, 9, and 3, respectively. Their allelic frequency was 56%, 72%, 13%, 29.5%, and 75.4%, respectively.

Both the normal and disease range of repeats in SCA vary in the population. According to the ACMG guidelines, the normal range of repeats is 6-36 for SCA1, 15-31 for SCA2, 12-40 for SCA3, 4-18 for SCA6, and 4-19 for SCA7, but different studies of the Indian population have reported varied ranges of repeats. A study by Alluri *et al*. (*10*) identified the normal range of repeats of SCAs in 187 samples. According to that study, the range of repeats was 20-37 for SCA1, 14-27 for SCA2, 6-38 for SCA3, and 3-20 for SCA7. The current study obtained a different normal range of repeats for SCA subtypes 1 and 7. A study by Saleem *et al*. (*9*) identified the normal range of repeats of SCAs in 150 samples. According to that study, the range of repeats was 7-37 for SCA1, 18-30 for SCA2, 14-37 for SCA3, and 9-14 for SCA7. In the current study, the range of repeats was 18-36 for SCA1, 19-31 for SCA2, 6-23 for SCA3, 3-18 for SCA6, and 3-19 for SCA7.

Looking at the size of repeats in different SCA subtypes in the Indian population indicates that the range of repeats varies. This may be because of the heterogeneous population in terms of ethnicity. Studies with a large sample and functional studies need to be conducted to redefine the range of repeats in the Indian population.

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Ethics approval

This study was approved by 103rd Institutional Ethics Committee (IEC) "2017-20-PhD-95 PGI/BE/282/2018" of SGPGIMS Lucknow. Subjects provided informed consent before being enrolled in the study.

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Brief Report

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Advantages of ddPCR in detection of *PLP1* duplications

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Summary **Pelizaeus–Merzbacher disease (PMD) is an X-linked, recessively inherited disorder associated with hypomyelination in the brain white matter. Mutations involving the proteolipid protein 1 gene (*PLP1*) located on Xq22.2 are responsible for PMD. *PLP1* duplication is the major genetic abnormality in PMD patients. In this study, we utilized droplet-digital polymerase chain reaction (ddPCR) as a potential method to detect *PLP1* duplications. Samples from four PMD patients and one of their mothers were used as positive controls. They had been previously diagnosed as having an additional *PLP1* copy by chromosomal microarray testing. Genomic copy number of *PLP1* was analyzed in triplicate experiments and compared with reference genes *XIST* and *AR* on the X-chromosome, and *RPP30* and *RPPH1* on the autosomes. As a result, precise results were obtained for each triplicate procedure. Thus, we concluded that triplicate experiments are no longer necessary. Compared to other methods, including fluorescence *in-situ* hybridization, multiplex ligation-dependent probe amplification, chromosomal microarray testing, and quantitative PCR, we were able to establish ddPCR results rapidly with very small amounts of DNA. In conclusion, we showed that ddPCR can be a potential diagnostic tool to confirm genomic copy number as a routine clinical application, including in prenatal diagnostic settings.**

Keywords: *PLP1* duplications, droplet digital polymerase chain reaction, copy number variations

1. Introduction

Pelizaeus–Merzbacher disease (PMD) is a genetic disorder associated with hypomyelination in brain white matter, and most patients with PMD exhibit motor developmental delay, hypotonia, horizontal nystagmus, and progressive spasticity (*1*). The proteolipid protein 1 gene (*PLP1*) located on Xq22.2 is responsible for PMD. Thus, PMD is recognized as an X-linked recessive disorder and most patients are male. Their *PLP1* abnormalities are often inherited from their carrier mothers. It is also known that two-thirds of PMD patients generally show chromosomal microduplications involving the Xq22.2 region, which harbors *PLP1* (*2*). For these reasons, it is recommended to first screen for *PLP1* duplication in cases of patients suspected of having PMD. In such cases, fluorescence in-situ hybridization (FISH) and multiplex ligation-dependent probe amplification (MLPA) methods have been used previously. The more efficient method is chromosomal microarray testing, because this method can detect not only duplications but also identify extent of duplications.

When we detected *PLP1* duplications in probands, diagnosis of carrier status of their mothers is often required. Furthermore, when mothers carry *PLP1* duplications, prenatal diagnosis is often required for subsequent pregnancies. In such cases, analytical methods need to fulfill certain conditions. Results should be rapidly and precisely obtained. It would be advantageous if the amounts of DNA required for these tests are small. Consequently, chromosomal microarray testing has limitations for the purpose of this screening.

The aim of this study was to establish a simple and rapid detection method to confirm *PLP1* duplications. With this aim, we utilized droplet-digital polymerase

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chain reaction (ddPCR) as a potential method as a 'proof-of-concept'.

2. Materials and Methods

2.1. *Materials*

Positive control samples were prepared as described in previous studies (*3*). Four PMD patients and one carrier mother were used. All patients had already been diagnosed to have *PLP1* duplications by chromosomal microarray testing. The sizes of the duplicated regions are shown in Table 1. Control samples from 1 normal male and 1 normal female were also used.

This study was performed in accordance with the Declaration of Helsinki and approved by the ethics committee of the Tokyo Women's Medical University. Written informed consent was obtained from patients and their parents before peripheral blood samples were acquired.

2.2. *Methods*

By use of the QIAamp DNA extraction kit (QIAGEN, Hilden, Germany), genomic DNA was extracted from peripheral blood samples. DNA concentration was calculated using a Qubit®2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA). *PLP1* was the target in this study. X inactive specific transcript (*XIST*), androgen receptor (*AR*), ribonuclease P/MRP 30kDa subunit (*RPP30*) and ribonuclease P RNA component H1 (*RPPH1*) genes were selected as references. *XIST* (Xq13) and *AR* (Xq12), were used as references for the X-chromosome. *RPP30* (10q23) and *RPPH1* (14q11) were used as reference for the autosomal chromosomes, as they are located in conserved regions, known to have low frequency of copy number variants (CNVs) (*4,5*).

Our priority in this study was to confirm the simplicity and accuracy of ddPCR. Because we used an intercalation system rather than TaqMan® hydrolysis probes, primers were not labeled with fluorescent dyes. We designed the primers with Primer3Plus (*https://primer3plus.com/*) and checked for homology using the UCSC Genomic Browser BLAT (*https://genome.ucsc.edu/cgi-bin/hgBlat*). Primers details are given in Table 2.

The ddPCR reaction mixtures were prepared as 20 µL total volumes, which included 10 µL 2 × QX200 ddPCR EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA), 1 µL each of the forward and reverse primers (1 µM), 1 µL DNA (20 ng/µL), and 7 µL RNase/DNase-free water. We loaded 20 µL of reaction mixture and 70 µL of QX200 Droplet Generation Oil for EvaGreen (BioRad Laboratories) into a QX100/200 DG cartridge (Bio-Rad Laboratories), which was transferred into a QX200 Droplet Generator (Bio-Rad Laboratories). Droplets containing 40 µL of oil and sample emulsion were transferred into clean 96-well PCR plates. To avoid contamination and evaporation, the plates were sealed using a PX1 PCR plate sealer (Bio-Rad Laboratories) with pierceable foil heat seals (Bio-Rad Laboratories). Then, PCR was performed with a Gene Amp PCR system 9700 (Thermo Fisher Scientific, Waltham, MA, USA). The thermal cycling conditions were as follows: 95℃ for 5 min (1 cycle); then 40 cycles of 95℃ for 30 s and 60℃ for 1 min; 4 ℃ for 5 min, 90℃ for 5 min, and then held indefinitely at 4℃. The ramp rate was 2℃/sec in all steps. After thermal cycling, droplets were analyzed for positive and negative signals using the QX200 droplet reader (Bio-Rad Laboratories). For each ddPCR sample, the same process was performed in triplicate. Data analysis was performed when the number of droplets produced was more than 10,000.

Table 1. Summary of the samples

Samples	Duplication sizes
Patient 1	648-Kb
Patient 2	603-kb
Patient 3	656-kb
Patient 4	948-kb
Carrier mother	603-kb
Control male	None
Control female	None

For data analysis, QuantaSoft Version1.7.4 software (Bio-Rad Laboratories) was used to statistically analyze the obtained data. Genomic copy number was calculated using the reference genes as follows: genomic copy number = (A/B) × C, where A is the concentration of the target DNA, B is the concentration of the reference DNA, and C is the number of copies of the reference gene. Because *RPPH1* and *RPP30* are located on the autosomal chromosomes, both are defined to have two copies. Copy numbers of *AR*, *XIST* and *PLP1* on the X-chromosome were different between males and females. Normal males and females are defined to have one and two copies, respectively.

3. Results and Discussion

Copy numbers of *AR*, *XIST*, and *PLP1* were calculated with two reference genes, *RPP30* and *RPPH1*, and obtained results were summarized in Table 3. Copy numbers of *RPP30* and *RPPH1* were calculated for each other as a reference. As shown, all replicates showed the copy numbers of *RPP30* and *RPPH1* close to "2", because these two genes are located on the autosomal chromosomes. In comparison, copy numbers of *AR* and *XIST* were different between males and females. Males and females showed the copy numbers of them as "1" and "2", respectively. It is reasonable because *AR* and *XIST* are located on the X-chromosome. From these findings, it was confirmed that ddPCR system can be used for detection of genomic copy number accurately.

In this study, *PLP1* duplications were targeted. Final

Table 2. Designs of the used primers

Targeted genes	Sense primers	Antisense primers	Product length (bp)
PLP1	5'-TCACAACCCCAAAGCAGCACATTTC-3'	5'-CGGCTAATTCAAAATCCAGCAAAGGG-3'	417
IRAK1	5'-AGCTCTGCATCATCGTCGT-3'	5'-CCAGCTTCTGGACCATCTTC-3'	76
XIST	5'-TGAGACCTGAGGACTGCAAA-3'	5'-AGCTTGGCCAGATTCTCAAA-3'	77
AR	5'-CCAGCAGAAATGATTGCACTA-3'	5'-CATTTCCGAAGACGACAAGA-3'	70
RPP30	5'-GATTTGGACCTGCGAGCG-3'	5'-GCGGCTGTCTCCACAAGT-3'	62
RPPH1	5'-GTCAGACTGGGCAGGAGATG-3'	5'-TGGCCGTGAGTCTGTTCC-3'	75

Table 3. Results of ddPCR

Samples		Versus RPPH1				Versus RPP30			
		AR	*XIST*	*RPP30*	*PLP1*	*AR*	*XIST*	*RPP30*	*PLP1*
Patient 1	1	1.08	1.10	2.14	1.84	1.00	1.04	1.86	1.72
	2	1.10	1.06	2.18	1.92	1.00	0.96	1.84	1.76
	3	1.04	1.02	2.08	1.82	1.00	0.98	1.72	1.76
Average		1.07	1.06	2.13	1.86	1.00	0.99	1.81	1.75
SD		0.02	0.02	0.03	0.03	0.00	0.02	0.04	0.01
Patient 2	1	1.22	1.02	2.20	2.04	1.10	0.94	1.82	1.86
	2	1.16	1.00	2.18	2.02	1.04	0.98	1.80	1.80
	3	1.20	1.14	2.32	2.10	1.02	0.98	1.72	1.88
Average		1.19	1.05	2.23	2.05	1.05	0.97	1.78	1.85
SD		0.02	0.04	0.04	0.02	0.02	0.01	0.03	0.02
Patient 3	1	1.02	0.98	2.02	1.76	1.02	0.98	1.98	1.74
	2	1.06	1.08	2.16	2.02	1.00	1.00	1.86	1.88
	3	1.14	1.02	2.14	1.98	1.06	0.96	1.86	1.84
Average		1.07	1.03	2.11	1.92	1.03	0.98	1.90	1.82
SD		0.04	0.03	0.04	0.08	0.02	0.01	0.04	0.04
Patient 4	1	1.10	1.14	2.12	2.02	1.04	1.08	1.88	1.90
	2	1.04	1.08	2.14	2.04	0.96	1.00	1.86	1.90
	3	1.04	1.06	2.16	1.90	0.98	0.98	1.86	1.76
Average		1.06	1.09	2.14	1.99	0.99	1.02	1.87	1.85
SD		0.02	0.02	0.01	0.04	0.02	0.03	0.01	0.05
Carrier	1	2.14	2.06	2.14	2.96	2.02	1.94	1.88	2.78
mother	2	2.08	2.04	2.08	3.00	2.00	1.96	1.92	2.88
	3	1.96	2.06	1.98	2.94	1.98	1.96	2.02	2.98
Average		2.06	2.05	2.07	2.97	2.00	1.95	1.94	2.88
SD		0.05	0.01	0.05	0.02	0.01	0.01	0.04	0.06
Male	1	1.00	0.92	2.06	1.00	0.96	0.88	1.86	0.96
control	2	1.04	1.04	2.18	1.00	0.96	0.96	1.84	0.92
	3	1.00	0.94	1.94	0.96	1.02	0.98	2.08	1.00
Average		1.01	0.97	2.06	0.99	0.98	0.94	1.93	0.96
SD		0.01	0.04	0.07	0.01	0.02	0.03	0.08	0.02
Female	1	2.06	2.04	2.02	1.96	2.04	2.02	1.98	1.94
control	2	2.08	2.10	2.10	2.00	1.96	1.98	1.90	1.90
	3	2.16	2.02	2.18	2.00	1.96	1.94	1.94	1.94
Average		2.10	2.05	2.10	1.99	1.99	1.98	1.94	1.93
SD		0.03	0.02	0.05	0.01	0.03	0.02	0.02	0.01

SD, standard deviation.

results are graphically summarized in Figure 1. As shown, all four male PMD patients demonstrated two copies of *PLP1* and the carrier females demonstrated three copies of *PLP1*. All triplicates showed similar results (Table 3), and there were no data which showed dispersed values. From these findings, each independent ddPCR result provided precise results.

There are several diagnostic methods to detect CNVs such as FISH, MLPA, chromosomal microarray testing, qPCR, and dPCR (*6*). At first, the capacity for precise detection of *PLP1* duplications should be considered. Previously, FISH has been used to detect *PLP1* duplications. In the case of FISH, detected signals are the result of hybridization of labelled DNA probes to corresponding chromosomal regions. Thus, detected signal numbers in a set of karyotype correspond to copy numbers. However, when the targeted regions are small, it is sometimes difficult to detect duplications on the same chromosome, because two signals cannot be separately detected or resolved (*3*). In this regard, interpretations of FISH results are often subjective.

Reaction conditions for ddPCR may be similar to qPCR. Traditional qPCR, based on relative quantification of target DNA, also can be used to detect

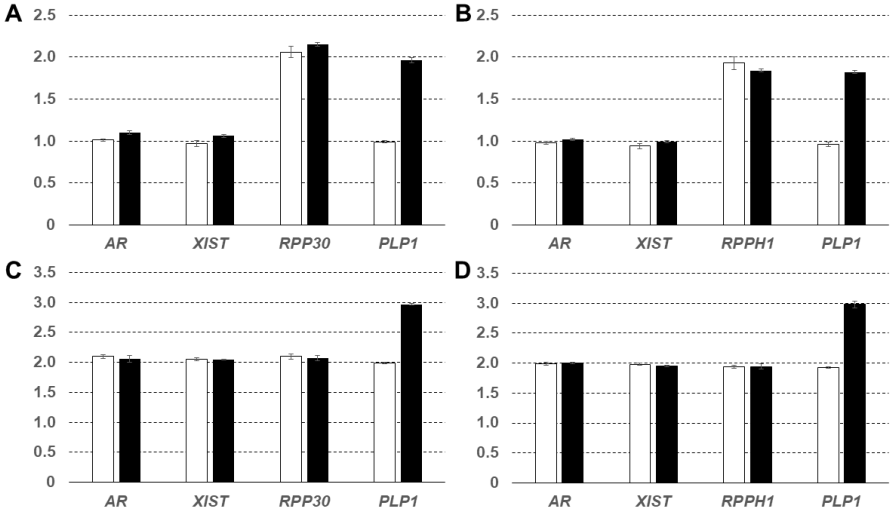

Figure 1. Graphical presentations of droplet digital PCR (ddPCR) results. Average copy number of the triplicates of the targeted genes versus reference genes are shown. Results of male patients (black; *n* = 4) and a normal male control (white; *n* = 1) were compared with *RPPH1* **(A)** and *RPP30* **(B)**. Results of a carrier female (white; *n* = 1) and a normal female control (black; *n* = 1) were also compared with *RPPH1* **(C)** and *RPP30* **(D)**. The Y-axis represents copy number estimated by ddPCR. Error bars indicate ± 2SD.

genomic copy number. Because qPCR is based on PCR amplification in which DNA is doubled in each amplification cycle, it is usually difficult to detect copy number gains of 1.5 ×. To compensate for unstable detection, independent triplicate experimental data sets are required for qPCR (*7,8*).

In comparison, we should be able to precisely detect small duplications by use of new technologies including MLPA and chromosomal microarray testing. In this study, ddPCR also provided precise results of *PLP1* duplications in PMD patients and the carrier mother. For this reason, ddPCR can be used in the same way as same as MLPA and chromosomal microarray testing.

The ddPCR method is a new technology that is based on partitioning template DNA and performing multiple independent PCR amplifications (*9*). In particular, the ddPCR system was developed to distribute template DNA randomly into emulsions of water-in-oil droplets (*10*). With data involving positive (containing target DNA) and negative (no target DNA) PCR amplifications, ddPCR provides absolute detection of genomic copy number and precise quantification of target DNA. In this study, results of all triplicates were the same for each sample. These results showed that ddPCR led to accurate CNV detection in one experimental pr°Cess, and triplicate experiments are no longer necessary.

Next, we wanted to evaluate assay time required. For FISH analysis, chromosome specimens are needed. For that, at least several days are required. Hybridization also requires time (generally, a number of days). For chromosomal microarray testing, long hybridization times are required (generally overnight). For MLPA, hybridization requires 16 hours. Thus, ddPCR has advantages with regard to required time. As shown, ddPCR requires thermal cycling reactions and droplet read-outs. For these experiments, approximately 6 hours are needed. Compared to other methods, we were able to obtain ddPCR results rapidly.

Furthermore, we wanted to consider required sample amounts. For chromosomal microarray testing, 250 ng is required when we use the Agilent CGH Microarray system (Agilent Technologies, Santa Clara, CA, USA). This is excessive in comparison with MLPA and ddPCR. MLPA generally requires 50-200 ng. ddPCR needs only 20 ng. In this regard, ddPCR also has an advantage.

In the case of prenatal diagnosis, carrier mothers will require the result as soon as possible. Regarding sample amounts, we can only extract a small amount of DNA from amniotic fluid (generally, less than 500 ng). Therefore, the necessary time needed and sample amounts required are the critical points for prenatal diagnosis. ddPCR will fulfill these demands with precise results. In conclusion, we showed that ddPCR is a potential diagnostic tool to confirm genomic copy number as a daily clinical application, including prenatal diagnosis.

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Brief Report

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Pre-Paget cells: Evidence of keratinocyte origin of extramammary Paget's disease

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Summary **Extramammary Paget's disease (EMPD) is a carcinoma of the genital, perianal, and, rarely, axillary skin. The malignant Paget cells migrate extensively in the epidermis before invading the underlying dermis. Toker cells and keratinocytes have both been suggested as the cells of origin of EMPD. Paraffin sections of eight cases of EMPD were immunohistochemically stained for carcinoembryonic antigen, a known marker for Paget cells. The presence of carcinoembryonic antigen in some keratinocytes in all of the observed cases of EMPD suggests that EMPD originates from keratinocytes. Thus, keratinocytes containing carcinoembryonic antigen are pre-Paget cells.**

Keywords: EMPD, Paget cells, carcinoembryonic antigen, pre-Paget cells, keratinocytes

1. Introduction

Paget's disease of the skin is a carcinoma that occurs as isolated cells and groups of cells called Paget cells which migrate extensively in the epidermis before invading the dermis (*1,2*). Paget's disease is most common in the skin of the nipple where it usually originates by migration of cells from a ductal carcinoma (*3*). Extramammary Paget's disease occurs in the genital, perianal, and, rarely, axillary skin, usually in the absence of an associated cancer (*4,5*). There is considerable evidence that extramammary Paget's disease is not be the same disease as Paget's disease of the breast (*6,7*).

Paget cells are readily distinguished from keratinocytes by their larger cytoplasmic volume and even larger nuclei (*1,8*). Most Paget cells also contain biochemical markers that distinguish them from keratinocytes (*6,9-11*).

Toker cells are variably present benign epithelial cells that are morphologically similar to Paget cells (*12*). The reported frequency of Toker cells in the normal epithelium of the external genitalia does not exceeded 36% (*13*). It has been suggested that extramammary Paget's disease originates from Toker cells (*14-16*) although they are present in only a few cases of extramammary Paget's disease (*17*). Like Paget cells, Toker cells usually contain cytokeratin 7 (*18*) and epithelial membrane antigen (*19*). Unlike Paget cells, Toker cells are negative for carcinoembryonic antigen (*19*). The reaction of Toker cells with antibody to the progesterone receptor depends on the antibody chosen (*12,18*). Paget cells are only rarely positive for the progesterone receptor (*20,21*).

In 1975, Bussolati and Pich found β-casein, a marker for Paget cells, in keratinocytes near Paget cells in both mammary and extramammary Paget's disease (*22*). They called the keratinocytes with Paget cell markers "pre-Paget cells". In 2008, Smith et al. found epithelial membrane antigen, another marker for Paget cells, in keratinocytes in a case of extramammary Paget's disease (*23*). Unaware of the earlier paper, they called the keratinocytes with Paget cell markers "incipient Paget cells". The name "pre-Paget cells" has priority and is more easily found by search engines.

Carcinoembryonic antigen is a reliable marker for extramammary Paget's disease (*11,19*) that does not require unmasking after formalin fixation (*24,25*). Since carcinoembryonic antigen does not occur in Toker cells (*19*), it might be the best marker for pre-Paget cells.

2. Materials and Methods

The Cooperative Human Tissue Network provided

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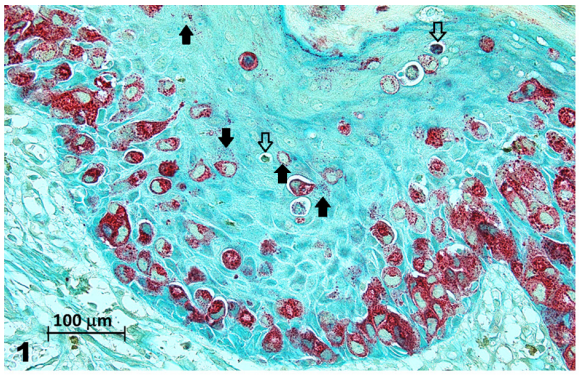

igure 1. Extramammary Paget's disease of the vulva stained for carcinoembryonic antigen with Vector Red and counterstained with fast green FCF. Several keratinocytes contain carcinoembryonic antigen (solid arrows), and two Paget cell do not (hollow arrows).

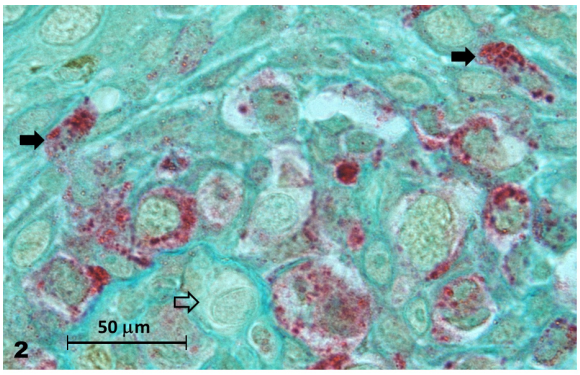

Figure 2. Extramammary Paget's disease of the vulva stained for carcinoembryonic antigen with Vector Red and counterstained with fast green FCF. Two keratinocytes contain carcinoembryonic antigen (solid arrows), and one Paget cell does not (hollow arrows).

unstained slides of formalin-fixed paraffin sections from 8 cases of extramammary Paget's carcinoma.

One slide from each case was incubated overnight at 4oC with 1/500 antibody to human carcinoembryonic antigen (Genetex rabbit polyclonal anti CD66e) followed by horse anti-rabbit IgG conjugated to calf intestine alkaline phosphatase via dextran ("ImmPressAP," Vector Labs), stained with Vector Red, and counterstained with 0.15% fast green FCF in 0.6% aqueous phosphomolybdic acid.

3. Results and Discussion

Carcinoembryonic antigen was present in all Paget cells in two cases and in most Paget cells in the other six cases (Figure 1 and Figure 2). Some keratinocytes in each case also showed staining for carcinoembryonic antigen (Figure 1 and Figure 2). There was no relation between the number of Paget cells in the section and the number of keratinocytes staining for carcinoembryonic antigen. In one case 15% of the cells staining for carcinoembryonic antigen were keratinocytes; in another case 10% were. In the other six cases 3% or fewer of the cells staining for carcinoembryonic antigen were keratinocytes.

Carcinoembryonic antigen is a reliable marker for Paget cells that is not found in normal keratinocytes. It follows that keratinocytes staining for carcinoembryonic antigen are pre-Paget cells.

The extreme variation in the ratio of pre-Paget cells to Paget cells is less surprising than it seems. The clinical course of EMPD is also extremely variable (*2,11,25*). Moreover, the response of EMPD to imiquimod varies from complete remission to complete resistance (*2,26*).

It is a priori unlikely that EMPD would originate from Toker cells which are usually absent in EMPD (*17*). For EMPD to have originated from Toker cells, all of the Toker cells in the tissue would have had to undergo malignant transformation at once.

The presence of a Paget cell protein in many keratinocytes in cases of EMPD strongly suggests that EMPD originates from keratinocytes. This also suggests that EMPD does not originate from Toker cells which do not contain carcinoembryonic antigen.

Paget cells often arrange themselves into a glandular pattern (*11*). They also share many antigens with the apocrine glands of the skin (*9,22,27*). EMPD may be a miscarried attempt to form apocrine glands.

The apparent presence of pre-Paget cells in EMPD suggests that this carcinoma does not originate from a single mutated cell, but from mutations in many cells. It further suggests that recruitment of new malignant cells is ongoing. Ongoing recruitment of malignant cells would explain other peculiarities of EMPD. EMPD has a high recurrence rate after apparent total excision (*4,5,11,25*). New foci of EMPD often appear at great distance from the original focus (*4,16,28,29*).

Acknowledgements

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Ethical Standards: The tissues in this study were removed in an effort to cure a life-threatening condition. Since the tissues were submitted to a tissue bank and sent to the author from the tissue bank, the author has no knowledge of the identity of the patients or the date of the surgery. The author knows only that the surgeries were performed in the eastern coastal states of the United States. This study was approved by Barry University's Institutional Review Board.

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Case Report

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Torcular herophili and lateral sinus thrombosis: An atypical presentation of Lemièrre's syndrome

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Summary **Lemièrre's syndrome (LS) is an uncommon disease characterized by septic thrombophlebitis of the jugular vein in the context of otorhinolaryngologic infections. These patients are often young and the pharyngotonsillar infection is the most frequent primary focus, but other foci like acute otitis media or otomastoiditis have been described. Although the internal jugular vein is the most commonly affected site, a few case reports have been published with thrombosis of other veins, such as the facial vein or transverse sinus. We report the case of a 93-year-old woman with an atypical presentation of LS presenting with thrombophlebitis of the internal jugular vein, transverse sinuses and Herophili torcula after an acute otitis media complicated with acute otomastoiditis. Infectious cerebral venous thrombosis (CVT) is rare and accounts for 6-12% of the total in large adult series and is usually associated to otorhinolaryngologic infections. CVT is an atypical presentation of LS that can be potentially lethal, especially during the acute phase. For this reason, clinical suspicion and early treatment are vital to improve the prognosis of these patients. Although surgical treatment is recommended in cases of LS complicated with CVT, conservative management with antibiotics and anticoagulation lead to ad integrum restitutio without neurological sequelae in our case, suggesting that surgical treatment may not be necessary in all cases of LS complicated with CVT.**

Keywords: Lemièrre's syndrome, torcula herophili, cerebral venous thrombosis, otomastoiditis

1. Introduction

Lemièrre's syndrome (LS) is an uncommon disease characterized by septic thrombophlebitis of the jugular vein in the context of otorhinolaryngologic infections, with an annual incidence of 3.6 cases per million people (*1,2*). The most commonly involved bacteria is *Fusobacterium necrophorum* but other bacteria such as fusobacteria, *Streptococcus*, *Staphylococcus*, and *Enterococcus* are commonly found in cultures. Although the internal jugular vein is the most commonly affected vein, a few case reports have been published with thrombosis of other veins, such as the facial vein or transverse sinus (*3-6*). In a recent systematic review of LS, a large proportion of cases had septic emboli in the lungs (*2*). Although less frequently, septic emboli were also found in other organs, such as the liver, spleen, joints, heart, and central nervous system. These patients are often young and the pharyngotonsillar infection is the most frequent primary focus, but other foci like acute otitis media or otomastoiditis have been described (*7*).

In the pre-antibiotic era, LS was associated with a case-mortality rate of 32-90%, with embolic events in 25%, and endocarditis in 12.5% (*8*). Currently, it is still a potentially life-threatening disease with a reported mortality of up to 17% (*1,2*). The recommended

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management includes several weeks of antibiotic therapy active against *Fusobacterium*, often combined with surgical drainage. However, the benefit of anticoagulation is uncertain. The recommended empirical antibiotic should include a beta-lactamase resistant beta-lactam antibiotic associated with metronidazole (*2,8*).

The purpose of this case is to present a case of LS complicated with cerebral venous thrombosis successfully managed with a conservative treatment that comprised anticoagulation and antibiotic therapy.

2. Case Report

A 93-year-old woman presented to the Emergency Department with a 48-hour history of left-sided neck swelling and pain. In the 3 weeks before admission, she noted purulent otorrhea through the left ear. She denied a recent history of fever or headache. Her past medical history was significant for hypertension, breast cancer diagnosed 30 years ago and currently in complete remission, pulmonary embolism 3 years before and paroxysmal atrial fibrillation. She did not take no other treatment. She was receiving oral levofloxacin for 2 days prior to admission.

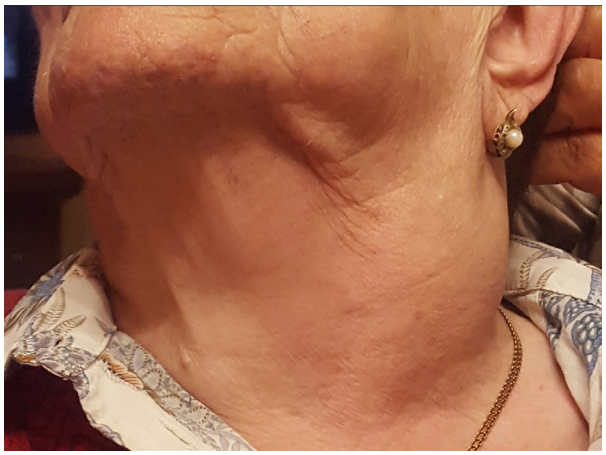

Figure 1. Physical exam revealed a left-sided neck swelling from the left submaxillary region to the supraclavicular region.

On admission, temperature was 36.4ºC, blood pressure 110/50 mm Hg and heart rate 72 bpm. A left-sided neck swelling from the left submaxillary region to the supraclavicular region (Figure 1) and deviated uvula with an area of an inflamed tonsil were noted. An otoscopy showed low secretion at left tympanic box level, slight hyperemia of the malleus and integrity of the tympanic membrane. The remainder of the physical examination was normal.

Laboratory tests disclosed hemoglobin 14.4 g/dL, leukocytes 9400/mm^3, fibrinogen 965 mg/dl, INR 1.29, C-reactive protein 20.8 mg/dL and ferritin 499 mcg/L. A computed tomography (CT) with contrast showed left side thrombosis including innominate trunk, extra and intracranial jugular vein, sigmoid sinus and transverse sinus, and partial thrombosis of torcular herophili and soft-tissue density material occupying the tympanic cage and left mastoid cells (Figure 2). A funduscopy did not show papilledema. A chest X-ray was normal. Empirical treatment was initiated with intravenous amoxicillin/clavulanic acid and anticoagulation with weight-adjusted enoxaparin. The patient presented a good response to initial therapy and showed a rapid improvement of local cervical inflammation. Bacterial identification was not performed. After 6 days, she was discharged and amoxicillin/clavulanic acid was continued for 3 weeks and anticoagulation for 3 months, without evidence of recurrence or complications.

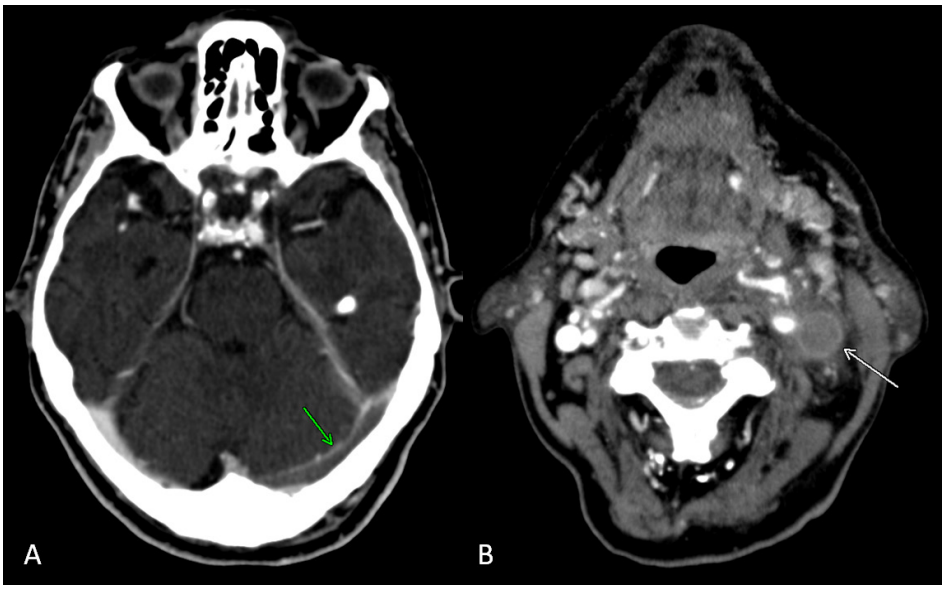

Figure 2. Combined sinus and jugular thrombosis. (A) A head CT scan shows a filing defect in the left transverse sinus (*green arrow*) and normal flow in the contralateral one. **(B)** The neck CT scan at the level of the oropharynx displays left internal jugular vein thrombosis (*white arrow*) and surrounding edema.

3. Discussion

Firstly described in 1936, LS is a septic thrombophlebitis of the jugular vein in the context of otorhinolaryngologic infections (*1*). However, the definition of LS still remains unclear in the literature. Some authors only include disseminated *F. necrophorum* infections originating from the throat, while others include all disseminated bacterial otorhinolaryngologic infections, as they have much in common, even though they may originate from different head foci and have different age distributions (*2*). LS with a primary otogenic focus predominantly occurs in otherwise healthy children, who mainly develop a spread of the infection into adjacent regions, e.g. mastoiditis and meningitis (*2*).

Our case of a 93-year-old woman represents an uncommon clinical presentation of LS since it occurred in an elderly patient and showed extensive intracranial progression of the thrombosis. In our case, surgical treatment was not considered due to the patient's age and comorbidities, along with the rapid improvement of the symptoms with conservative treatment. The evidence available regarding LS complicated with cerebral venous thrombosis (CVT) is scarce, and mostly based in case reports or case series (*3-6*). In one of the largest case series of infection-associated CVT, all patients were treated with antibiotics combined with local surgical drainage or resection of the infected site; however, 50% of these patients showed an unfavourable outcome (*5*). In a case series of infectious CVT in pediatric population, all cases were treated with surgery and antibiotic therapy, and the reported outcome was favourable in all cases, although almost half of patients developed mild hearing impairment (*4*). In our case report, despite the conservative treatment, outcome was favourable, suggesting that surgical treatment may not be necessary in all cases of LS complicated with CVT.

F. necrophorum is still the major microbiological agent identified in LS. Other microbiological agents include *Streptococcus*, including *methicillin resistant S. aureus*, and *S. aureus*. However, a large proportion of the cases have not reported a microbiological agent. As with other bacteria, cultures can be falsely negative if antibiotics are administered before sample collection (*7*). In our case, blood cultures were not obtained because the patient was afebrile and she had received antibiotics prior to admission.

Clinically, LS begins as an oropharyngeal infection with odynophagia, cervical lymphadenopathy and peritonsillar abscess (*9*). Then, usually 1 to 3 weeks later, the infection spreads to the parapharyngeal space and jugular vein, disseminating directly or via the peritonsillar venous plexus. This causes thrombosis that often manifests as a tender and swollen cord anterior to the sternocleidomastoid muscle. Other complications are carotid artery rupture, Horner's syndrome, paralysis of the trapezius muscle and dysphagia. Septic thrombophlebitis of the jugular vein is the primary source of emboli, most commonly to the lungs, but also to the joints, soft tissues, liver, spleen, kidney and central nervous system. It is in this latter category where our case can be included (*7*).

CVT is an uncommon condition with an incidence of 1.3-1.6/100,000 in Western countries (*10*). Traditionally, the most prevalent aetiology of CVT was infections but nowadays aseptic CVT are more frequent. Infectious CVT accounts for 6-12% of the total in large adult series, mainly in developing countries. The most common infections associated with CVT are otitis, mastoiditis and sinusitis. *F. nucleatum* and *F. necrophorum*, normal flora of the oral cavity, are the most common pathogens involved in infectious CVT (*11*).

Severe headache is the most common presenting symptom of CVT. A minority of patients (10%) do not report headache at baseline, as in our case. Other typical CVT symptoms are seizures, focal deficits, intracranial hypertension (decreased visual acuity and papilledema) and diffuse encephalopathy (*12*). The confirmation of the diagnosis of CVT depends on the demonstration of thrombi in the cerebral veins or sinuses. Three imagining techniques can be used: magnetic resonance imaging (MRI) with MR venography, CT venography and angiography (*13*). MRI is the most sensitive technique and its sensitivity is even higher if venography is associated. CT-venography is a decent and less expensive alternative for the diagnosis of CVT, but it is inferior for the visualization of brain parenchymal lesions that can appear in this situation, such as intracerebral, subdural or subarachnoid haemorrhage (*12*). In our case, a contrast CT was performed showing thrombi in several cerebral structures, including the torcular herophili, site of the confluence of superior sagittal, straight, occipital and transverse sinuses.

A routine blood test including a chemistry panel, complete blood count and coagulation study should be performed in all patients with CVT. D-dimer measurements are not used frequently in the diagnostic work-up of patients with suspected CVT due to its limited sensitivity (*12*). In patients with infectious CVT, laboratory markers of infection should also be measured.

Management of LS should include these general principles: systemic antibiotic therapy, drainage of abscesses, and consider anticoagulation (*6,7*). The optimal antibiotic regime for this pathology is unknown, as the evidence is scarce. Normally, *F. necrophorum* is reported to be susceptible to penicillin, cephalosporins, metronidazole, clindamycin, tetracyclines and chloramphenicol. B-lactamase-producing strains of *F. necrophorum* have only rarely been reported (*8*), and still no resistant strains have been found in Europe. In our case, empiric treatment with amoxicillin/clavulanic acid was used, with an excellent outcome.

Anticoagulation is normally not advised in LS, and it has been reserved for cases of thrombosis progressing

retrogradely to the cavernous sinus, according to the recommendations of international guidelines; a course of intravenous heparin followed by up to 3 months of treatment with oral coumarin to reduce morbidity among survivors and to allow adequate collateral circulation has been recommended. The internal jugular vein does not usually recanalize after resolution of the infection (*8*). Endovascular treatment using intrasinus thrombolysis or mechanical thrombectomy should not be routinely used in patients with CVT (*12*).

Patients with the otogenic variant of LS may have a less severe course than oropharyngeal LS, as long as they remain at the mastoiditis stage and do not develop meningitis, which is reported to have a mortality of up to 31.5% (*2*). CVT usually has a good prognosis (*13*), but severe cases can result in death or permanent disability. Approximately 5% of patients die in the acute phase of the disorder, being transtentorial herniation secondary to a haemorrhagic lesion the main cause of death. Status epilepticus, medical complications and pulmonary embolism are among the other causes of early death. Deaths after the acute phase are due to the underlying conditions or to side effects of prolonged anticoagulant treatment. Prognosis is less favourable in patients at both extremes of age.

In conclusion, we report a case of LS complicated with infectious cerebral venous thrombosis in an elderly patient. This uncommon condition can be potentially lethal, especially during the acute phase. For this reason, clinical suspicion and early treatment are recommended. Surgical treatment, along with antibiotics are the treatment of choice. Anticoagulation should be considered. In our case, evolution was favourable with conservative medical treatment, suggesting that surgery may not be necessary in all cases of LS complicated with CVT.

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Case Report

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Pituitary incidentaloma diagnosed as acromegaly triggered by trauma: A case report

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Summary **Pituitary incidentaloma (PI) is a generic term for pituitary tumors that are identified on images acquired for non-malignant conditions. Acromegaly is an extremely rare form of PI. Occasionally, a functional pituitary adenoma (PA) may be misdiagnosed as PI, which may result in a poor clinical outcome. Here we report the first case, to the best of our knowledge, of PI diagnosed as trauma-triggered acromegaly. A 42-year-old man with a chief complaint of head trauma was referred to our hospital after computed tomography (CT) revealed a pituitary tumor. His appearance was suggestive of acromegaly. Mild hypertrophy of the extremities was also observed. Preoperative blood tests, magnetic resonance imaging (MRI), and endocrine tolerance test findings indicated acromegaly. Accordingly, we suspected a growth hormone (GH)-producing PA, and we performed endoscopic transsphenoidal surgery (eTSS). Histopathology showed a densely granulated GH-producing PA, which was also confirmed via immunohistochemistry. Two months after surgery, blood tests showed decreased levels of GH and insulin-like growth factor-1. In addition, a postoperative endocrine tolerance test revealed no abnormalities. There was no recurrence at 24 months after surgery. The findings from this case suggest that PIs can also present as functional adenomas, which can be diagnosed using initial hormone examinations and endocrine tolerance tests. Therefore, thorough endocrine examination is necessary for early diagnosis and treatment and improved patient outcomes.**

Keywords: Acromegaly, pituitary incidentaloma, trauma

1. Introduction

Pituitary incidentaloma (PI) is a generic term for pituitary tumors that are first identified on images acquired for reasons unrelated to tumors, such as headache, trauma, or symptoms involving the neck or central nervous system (*1-4*). The wide application of sensitive brain imaging techniques such as computed tomography (CT) and magnetic resonance imaging (MRI) has led to increased recognition of such lesions. Although the etiology of PIs covers a wide range of pathologies [most of them (approximately 90%) are benign adenomas], they may result in visual and/or neurological abnormalities.

By definition, micro-incidentalomas have a maximum diameter of < 1 cm, while the diameter of macro-incidentalomas is at least 1 cm. Micro-incidentalomas have a reported mean prevalence of approximately 10% in normal individuals (*1*). Among PIs, growth hormone (GH)-producing pituitary adenomas (PAs) and acromegaly are extremely rare, with a prevalence of only 0.14% (*2*). Acromegaly is a rare disease, largely caused by GH-producing pituitary adenomas. However, its incidence is higher than previously believed, and early diagnosis is essential as there is an increased risk of morbidity and mortality when inappropriate treatment is given. Screening is recommended for all patients with clinical features of

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Table 1. Blood and endocrine test results for a 42-year-old man with a pituitary incidentaloma diagnosed as acromegaly triggered by trauma

white blood cell count	$5.6 \times 10^3/\mu L$
red blood cell count	$495 \times 10^4/\mu L$
hemoglobin	14.6 g/dL
hematocrit	45.7%
platelet count	$25.2 \times 10^4/\mu L$
Na	141 mmol/L
K	3.8 mmol/L
Cl	10^4 mmol/L
HbA1c	6.0%
LDL	86 mg/dL
C-reactive protein	0.09 mg/dL
D-dimers	< 0.3 μg/mL
Endocrine tests:	
growth hormone (GH)	4.97 ng/mL
insulin-like growth factor-1 (IGF-1)	426 ng/mL
luteinizing hormone (LH)	1.76 mIU/mL
follicle-stimulating hormone (FSH)	3.70 mIU/mL
adrenocorticotropic hormone	20.4 pg/mL
prolactin (PRL)	8.46 ng/m
anti-diuretic hormone	0.8 pg/mL
thyroid-stimulating hormone (TSH)	1.08 μIU/mL
free thyroxine (FT4)	1.05 ng/dL
free triiodothyronine (FT3)	17.9 pg/L
cortisol	6.96 μg/dL.

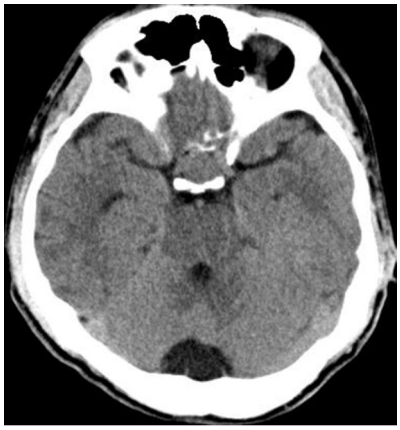

Figure 1. Brain computed tomography (CT) findings at initial presentation of a 42-year-old man with a pituitary incidentaloma. The image shows a pituitary tumor in the sella.

excess GH production. There is increasing knowledge that the classical acromegaly diagnostic criteria can no longer be applied broadly to everyone; some patients can have GH excess with a normal GH response to glucose. Treatment is multifactorial, and personalized therapy is thus advised (*5*).

Here, we report the first case, to our knowledge, of PI diagnosed as trauma-triggered acromegaly and present a review of the relevant literature.

2. Case Report

A 42-year-old man presented with a chief complaint of head trauma. He had no past history of tumors, head trauma, or other factors related to the development of a pituitary tumor. We performed head CT, which identified a pituitary tumor. He was admitted to our hospital in a fully conscious condition. His blood pressure and body temperature were 110/62 mmHg and 36.2°C, respectively. His height and weight were 170 cm and 64.5 kg, respectively. Heart and respiratory sounds were normal, and there were no neurological deficits. His heart rate was 62 beats/min. He exhibited an acromegaly-like appearance, and there were no obvious differences between his current appearance and that on his driver's license photograph taken 5 years prior. He also exhibited mild hypertrophy of the extremities, although there was no tongue enlargement or complaint of sleep apnea.

Blood and endocrine test results are outlined in Table 1. Electrocardiography and upper and lower gastrointestinal examinations revealed no abnormal findings. Head CT showed a slightly dense tumorous lesion that progressed from the intrasellar to the suprasellar regions (Figure 1). Radiography showed mandibular protrusion, flower cabbage-like deformities in the distal phalanges of the fingers, and 24-mm-thick soft tissue on the bilateral heel pads (Figure 2A, B, C). Brain MRI showed a tumorous lesion on the pituitary gland (maximum size: 22 mm), which exhibited a strong signal on gadolinium (Gd)-enhanced T1-

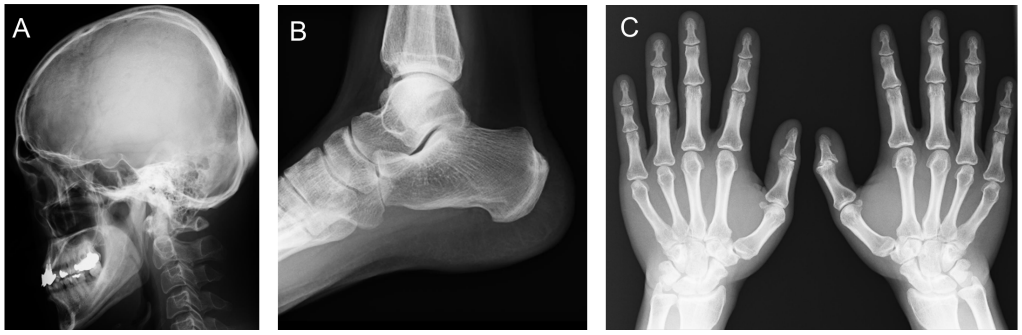

Figure 2. Morphological characteristics observed by radiography for a 42-year-old man with a pituitary incidentaloma. (A) A lateral skull radiograph shows mandibular protrusion, occipital nodule protrusion, skull thickening, and ballooning of the sella. **(B)** A foot radiograph shows thickening of the heel pad. **(C)** A hand radiograph shows lower cabbage-like, hypertrophic deformities in the distal phalanges.

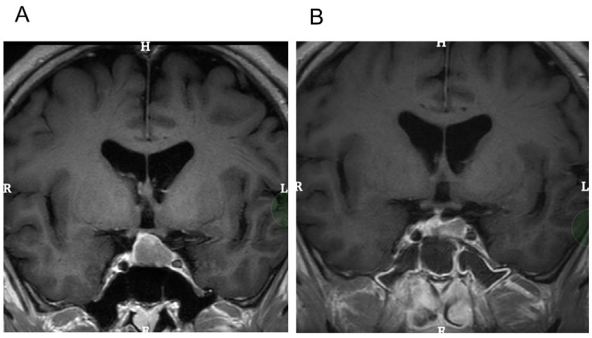

Figure 3. Gadolinium-enhanced T1-weighted magnetic resonance imaging (MRI) findings of a 42-year-old man with a pituitary incidentaloma. (A) An image obtained at presentation showing a pituitary tumor. **(B)** A postoperative image showing complete disappearance of the tumor.

weighted imaging (T1WI; Figure 3A).

2.1. *Preoperative endocrine tolerance test*

The corticotropin-releasing hormone (CRH) loading test revealed a paradoxical reaction of GH. The thyroid releasing hormone (TRH) loading test also yielded a paradoxical reaction of GH and a normal reaction of thyroid stimulating hormone (TSH). The luteinizing hormone-releasing hormone (LH-RH) loading test yielded normal reactions of GH, while the growth hormone releasing peptide-2 (GHRP-2) loading test yielded a normal reaction of GH and prolactin (PRL). The 75-g oral glucose tolerance loading test yielded a paradoxical reaction of GH, indicating the absence of diabetes. The bromocriptine and octreotide loading tests yielded a paradoxical decline in GH.

2.2. *Post-hospitalization course*

The patient's symptoms satisfied the diagnostic criteria for acromegaly, and endoscopic transsphenoidal surgery (eTSS) was performed. After incising the dura mater at the bottom of the sella, we performed extracapsular extraction followed by gross total resection. No cerebrospinal fluid leakage was observed during eTSS, and the surgery was completed after reconstruction of the bottom of the sella. Permanent pathology revealed a densely granulated, GH-producing PA, and immunohistology revealed GH-positive intracellular staining (Figure 4A, B).

2.3. *Postoperative course*

After surgery, we did not observe recurrence of the tumor or intraoperative hemostasis on MRI (Figure 3B). There were no complications such as cerebrospinal fluid leakage or diabetes insipidus. The patient was prescribed hydrocortisone (15 mg/day) and was discharged. Two months later, he underwent an endocrine tolerance test, which yielded GH and IGF-1 levels of 0.41 and 156 ng/

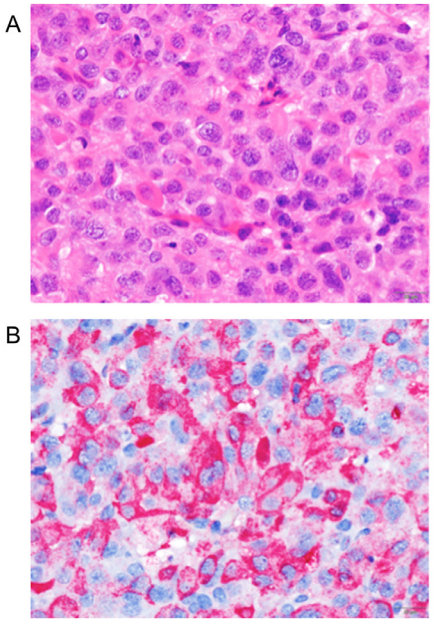

Figure 4. (A) Permanent pathology reveals a densely granulated, GH-producing PA (hematoxylin and eosin stain; H&E×60), and (B) immunohistology reveals GH-positive intracellular staining (GH×60).

mL, respectively. Thus, the patients' hormone levels had normalized relative to preoperative baseline levels. The CRH loading test yielded a paradoxical reaction of GH, while the TRH, LH-RH, and GHRP-2 loading tests yielded normal reactions. All other test results were normal. Twelve months after surgery, the patient underwent endocrine blood sampling which revealed further decreases in GH and IGF-1 levels (0.32 and 112 ng/mL, respectively). MRI revealed no recurrence, and hydrocortisone administration was terminated. The patient is currently being followed as an outpatient.

3. Discussion

A GH-producing adenoma is classically diagnosed based on a combination of increased GH levels, unsuppressed GH levels after a 75-g oral glucose tolerance test, and increased insulin-like growth factor (IGF)-1 levels. In our case, the 75-g oral glucose tolerance test had a bottom value of 1 or more, and the patient was diagnosed with clinical acromegaly from the 75-g oral glucose tolerance test and his acromegaly facial features.

Acromegaly can be promptly and easily diagnosed. The most common cause is a GH-producing PA, and the disease is characterized by facial features such as frontal bossing, thick eyelids, a large triangular nose, a thickened lower lip, and macroglossia; hypertrophy of the extremities; bone deformities; hyperhidrosis; and headache. Acromegaly is often also accompanied by impaired glucose tolerance, hypertension, metabolic disorders such as dyslipidemia and goiter, colon polyps, and malignant tumors (particularly colon cancer). Disease outcomes and life expectancy deteriorate

when it is untreated; therefore, early diagnosis and treatment are crucial (*6,7*). The patient in the current case was diagnosed with acromegaly on the basis of accentuated facial features, imaging findings, hormone tests, and endocrine tolerance tests. In a previous study that investigated 71 cases of PI, the overall mean age of patients was 51.6 years. The most common chief complaint was headache, and CT was used to detect the tumors (detection rate: 63.2%). Moreover, 17.6% patients had symptoms that facilitated early diagnosis. The diagnoses included pituitary adenoma (48 cases, 70.6%), Rathke's cleft cyst (RCC; nine cases, 3.2%), prolactinoma (five cases, 7%), and GH-producing PA (one case, 1.4%). In addition, 14 cases (20%) were diagnosed after hormone evaluations. Twenty-one patients (28.8%) underwent surgery according to the guidelines for PI treatment in Japan (*8*). This indicates that approximately 30% of PIs were indicated for surgery. Our patient was relatively young (42 years) and diagnosed with a GH-producing PA triggered by trauma, which is considered a rarity. Early diagnosis and treatment of PIs are important as functional adenomas, including acromegaly, may result in poor prognosis when there is a delay in diagnosis and treatment.

As far as we could find, based on a search of PubMed, this is the first case of PI diagnosed as acromegaly triggered by trauma (*1-4*). At the initial visit, the patient did not present symptoms suggesting PI or acromegaly. Early diagnosis and treatment resulted in complete remission in our case. Because PIs can become adaptive after surgery and patients can present with newly growing adenomas, regular endocrine tolerance tests, basal hormone value measurements, neurological and physical examinations, and visual acuity and visual field examinations during follow-up visits are essential. We also plan to monitor our patient over the long term.

In conclusion, the findings of this case suggest that PIs can also present as functional adenomas, which can be initially diagnosed by hormone assessments and endocrine tolerance tests. Thus, it is important to perform these tests in case of abnormalities in basal hormone values. This will help in early diagnosis and treatment, which can ultimately improve patient outcomes.

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Case Report

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Two-staged biliary reconstruction with temporary complete external biliary drainage as a bailout procedure in a pediatric patient after difficult living donor liver retransplantation

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Summary **Biliary leakage at the site of the hepaticojejunostomy after liver transplantation is a life-threatening complication. We herein present the case of a 7-year-old girl who underwent complete external biliary drainage during difficult living donor liver retransplantation as a bailout procedure. The patient had undergone duct-to-duct biliary reconstruction in the initial living donor liver transplantation. In the retransplantation, Roux-en-Y (RY) reconstruction was planned but abandoned due to the critical condition in the operation. As an alternative procedure, the patient underwent complete external drainage using a 6Fr drainage tube with cuff. Five months after retransplantation when the nutrition status and physical strength of a patient recovered fully, RY hepaticojejunostomy was successfully performed. This is a case report of two-staged biliary reconstruction with temporary complete external biliary drainage used in pediatric liver retransplantation, which was performed after some months not a few days. It is a safe and feasible alternative when primary anastomosis is deemed to carry a high risk of bile leakage in cases of difficult liver transplantation in critically ill patients.**

Keywords: Living donor liver transplantation, two-staged biliary reconstruction, Roux-en-Y hepaticojejunostomy

1. Introduction

Retransplantation, an extended operation time, severe malnutrition, and hepatic artery thrombosis have been reported as risk factors of biliary leakage, which may lead to fatal consequences (*1-4*). In addition, Roux-en-Y (RY) hepaticojejunostomy in retransplantation cases may require extensive adhesiolysis, putting the patient at an increased risk of bile leakage (*5*). Delayed biliary reconstruction after a few days from liver transplantation (LT) were reported to avoid initial biliary anastomosis in the literature (*6-9*). However, the patient's condition doesn't always recover fully to allow us to perform biliary reconstruction for a few days. We herein report a patient who underwent successful two-staged biliary reconstruction after five months from retransplantation.

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2. Case Report

The patient was a 5-year-old girl (height 98 cm and weight 14 kg). She underwent living donor liver transplantation (LDLT) for primary sclerosing cholangitis (PSC) with a left lateral segment graft donated from her 40-year-old father. Duct-to-duct (DD) reconstruction was performed. However, she suffered two episodes of biopsy-proven acute cellular rejection at three and nine months after LDLT. Her serum bilirubin level increased gradually two years after transplantation, and endoscopic retrograde cholangiopancreatography showed strictures of the intrahepatic biliary tract compatible with recurrent PSC. Her condition eventually progressed to decompensated liver cirrhosis. Her pediatric end-stage liver disease (PELD) score was 43.

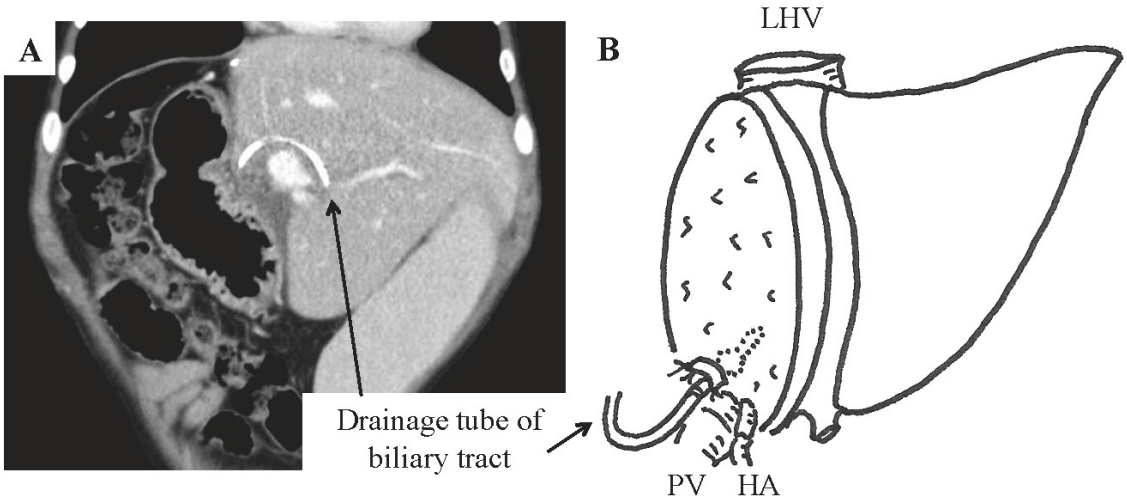

Figure 1. Computed tomography scan and schematic diagram of the lateral liver graft. (A) Temporary complete external biliary tube into the lateral liver graft. **(B)** Similarly, external biliary tube into the lateral liver graft.

She underwent retransplantation with a left lateral liver segment donated by her mother. Laparotomy revealed massive ascites and dense adhesion between the upper jejunal loops and the cirrhotic liver. The primary liver graft was explanted six hours after the skin incision. The new graft was placed, and hepatic and portal veins were reconstructed, after which the graft was successfully reperfused (warm and cold ischemic time, 37 and 176 minutes, respectively). Microvascular hepatic arterial reconstruction followed. At this time, 8 hours had passed since the skin incision, and the estimated blood loss was over 6,000 g. The total urine output was only 50 ml, suggesting significant intravascular volume depletion, and her hemodynamics were unstable despite the use of catecholamine.

The native common bile duct was severely injured during primary liver graft removal, and DD re-reconstruction was impossible. In addition, the intestine was diffusely edematous and was unsuitable for biliary reconstruction. We decided to perform external biliary drainage instead of primary anastomosis. A 6-Fr drainage tube was placed in the left hepatic duct of the liver graft, measuring 3 mm in diameter (Figure 1). The orifice was closed using interrupted sutures of 5-0 prolene and the tube was fixed to the bile duct with a single tie. The tube was passed through the abdominal wall as an external biliary drainage route.

After the operation, she required an intensive-care unit stay of one month, including two weeks on machine ventilation. Enteral nutrition and gait rehabilitation were started on the postoperative day 5 and 31, respectively. Her general condition eventually recovered thereafter. She and her family were given detailed instructions to safely manage the external biliary drainage tube at home, and she was discharged three months after retransplantation with an Eastern Cooperative Oncology Group Performance Status (ECOG PS) of 3. Our transplant coordinators maintained close contact with the patient and her family to make sure that there were no tube-related complications. At five months after retransplantation, she was confirmed to have fully recovered from her impaired nutritional status with a weight gain of 2 kg and an ECOG PS of 1. She underwent elective hepaticojejunostomy with an uneventful postoperative course and was discharged 25 days after the operation. Since then, she has been doing well for eight years without biliary complication or signs of recurrent PSC and liver function test has been within normal level.

3. Discussion

We herein report the first pediatric patient who underwent two-staged biliary reconstruction after some months from reLDLT. Several centers have previously reported the usefulness of delayed biliary reconstruction after LT (*6-8*). Komorowski *et al*. (*7*) reported that perihepatic packing and temporary abdominal closure with delayed biliary reconstruction were viable options for massive uncontrollable bleeding and bowel edema during LT. DiNorcia *et al*. (*9*) reported on 150 adult patients including retransplantation (29.3%) with postreperfusion hemodynamic instability and coagulopathy during LT which required damage control. Eighty-four of the 150 underwent delayed biliary reconstruction. In both reports, as well in others, the interval between LT and biliary reconstruction was approximately two days, during which hemostasis was achieved with stable vital signs and the general condition of the recipient was deemed fit for relaparotomy (*6,7,9*). However, our patient was considered too sick to bring back to the operating room at only a few days after reLDLT, as demonstrated by her one-month stay in the intensive-care unit. We therefore decided to postpone biliary reconstruction until her nutritional status and physical

strength had fully recovered. To minimize the risk of tube-related complications, such as accidental tube dislodgment and cholangitis, which in her case was considered higher than in adults, she and her family were given strict instructions from the nurses regarding tube management at home. Our transplant coordinators were also available 24 hours a day, 7 days a week, in case of emergency and made frequent phone calls to make sure that the patient was safe. Such meticulous multidisciplinary management enabled us to safely perform two-staged biliary reconstruction in a pediatric patient.

Indications for two-staged biliary reconstruction should be based on variable factors, including the preoperative condition (*e.g.*, high PELD/MELD, ECOG PS, nutritional status, retransplantation) and difficulty of the operation (*e.g.*, duration, estimated blood loss, hemodynamic instability requiring high doses of vasopressive agent, anuria, level of metabolic acidosis, significant bowel edema, quality of the liver graft). The timing of the biliary reconstruction should also be determined on a case-by-case basis. Complete functional recovery with a good nutritional condition is essential for successful staged surgery. In LDLT, the graft functional recovery tends to be slower than that after deceased donor LT, so the timing of relaparotomy should be set later. Although there is no general rule regarding the appropriate timing of staged biliary reconstruction in critically ill patients, it may be worthwhile to wait several months. In fact, the feasibility of two-staged pancreaticojejunostomy at three months after pancreaticoduodenectomy for patients who are at a high risk of pancreatic fistula has been reported (*10*). In the present case, staged biliary reconstruction was delayed up to five months after retransplantation because of the patient's poor nutritional and functional status. Despite this delay, this surgical procedure was safely performed five months after retransplantation without severe adhesion.

In conclusion, two-staged biliary reconstruction with temporary complete external biliary drainage is a safe and feasible alternative in difficult LT in critically ill patients.

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Case Report

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Effectiveness of endoscopic transsphenoidal surgery for gonadotroph adenoma mimicking dementia: A case report

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Summary **There are few reports of pituitary adenomas (PA) mimicking dementia. Delay in disease diagnosis and treatment may result in poor clinical outcome. We experienced a rare case where endoscopic transsphenoidal surgery (eTSS) effectively treated a gonadotroph adenoma mimicking dementia and report on literature considerations. We report the case of a 72-year-old man with chief complaints of cognitive decline, bradykinesia, anorexia, dressing apraxia, and vigor decline over several months. He was admitted to our hospital for scrutiny in a disoriented state. Blood tests showed hyponatremia and thyroid hormone depression. Magnetic resonance imaging showed a pituitary tumor, and preoperative endocrine stress tests showed reduced reactivities of growth hormone, adrenocorticotropic hormone/cortisol, and luteinizing hormone/follicle-stimulating hormone. Symptomatic pituitary adenoma was suspected, and eTSS was performed. The permanent pathological diagnosis was of gonadotroph adenoma. Postoperatively, the hyponatremia, cognitive decline, movement retardation, loss of appetite, dressing apraxia, and limb edema markedly improved. The patient was discharged under hydrocortisone 15 mg/day administration without complications. The endocrine stress test performed 2 months postoperatively showed secondary hypoadrenocorticism, while the other endocrine functions had normalized. No recurrence had occurred by 30 months postoperatively; the medication of hydrocortisone was gradually discontinued and the patient at the time was still being followed as an outpatient with modified Rankin Scale score 0. Secondary hypothyroidism and secondary hypoadrenocorticism due to the pituitary tumor primarily caused the condition. It is important to consider PA in the differential diagnosis of dementia, and early diagnosis and treatment can contribute to a patient's good clinical outcome.**

Keywords: Pituitary adenoma, endoscopic transsphenoidal surgery, dementia, hypoadrenocorticism, cognitive decline

1. Introduction

There are few reports of pituitary adenomas (PAs) mimicking dementia, *e.g*., Brisman *et al*. (*1*) reported a case of reversible dementia due to macroprolactinoma. The case involved a patient with a huge prolactinoma who came to medical attention because of dementia. The tumor shrank dramatically after bromocriptine therapy, and the patient's mental status returned to normal (*1*). Subfrontal tumors are an infrequent cause of dementia (*1*). A giant pituitary adenoma can be easily identified as a cause of dementia, but it may be overlooked if it is a localized lesion in the sella.

Delay in disease diagnosis and treatment may result in poor clinical outcome. We experienced a rare case where endoscopic transsphenoidal surgery (eTSS) effectively treated a gonadotroph adenoma, at the localized sellar lesion, mimicking dementia and report

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on literature considerations.

2. Case Report

A 72-year-old man with chief complaints of cognitive decline, bradykinesia, slow thinking, anorexia, dressing apraxia, and vigor decline over several months and past history of hypertension and dyslipidemia was admitted to our hospital for scrutiny. On admission, he was disoriented and had a blood pressure and body temperature of 110/62 mmHg and 36.2 °C, respectively. His height was 170 cm, and his weight was 64.5 kg. His heart and respiratory sounds were normal and he had no neurological deficits; his heart rate was 62 beats/min. Blood tests showed hyponatremia (Na, 127 mEq/L) and cortisol depression (adrenocorticotropic hormone (ACTH), 9.3 pg/mL; cortisol, 0.69 μg/dL) and thyroid hormone depression (thyroid-stimulating hormone (TSH), 1.78 ng/mL; free thyroxine, 0.78 ng/mL; free triiodothyronine, 2.22 ng/mL) were noted. We performed head magnetic resonance imaging (MRI) to investigate the dementia symptoms and detected a pituitary tumor (maximum size, 18 mm), with a strengthened signal on gadolinium (Gd)-T1-weighted imaging (Figure 1).

The blood and endocrine test results are outlined in Table 1, which shows that there were reduced reactivities of ACTH/cortisol and luteinizing hormone/follicle-stimulating hormone (LH/FSH).

2.1. *Preoperative endocrine tolerance test*

The corticotropin-releasing hormone (CRH) loading test revealed reduced reactivity of ACTH/cortisol and growth hormone (GH). The thyroid releasing hormone (TRH) loading test showed normal reactivity of TSH and reduced reactivity of GH. The LH-releasing hormone (LH-RH) loading test showed reduced reactivities of LH/FSH. The growth hormone releasing peptide-2 loading test showed normal reactivity of GH and prolactin.

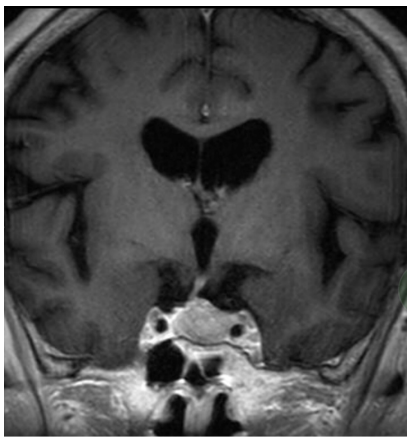

Figure 1. Head gadolinium-T1-weighted magnetic resonance imaging at first visit shows a pituitary tumor in the sella.

2.2. *Hospitalization course*

Symptomatic PA was suspected, and eTSS was performed. After incising the dura mater at the bottom of the sella, extracapsular extraction was performed, and gross total resection was achieved. No cerebrospinal fluid leakage was observed during the eTSS, and the surgery was completed after reconstruction of the bottom of the sella. Permanent pathology revealed a gonadotroph adenoma, and immunohistology revealed LH/FSH-positive intracellular staining (Figure 2).

2.3. *Postoperative course*

Postoperatively, we did not observe recurrence of the tumorous lesion on MRI (Figure 3). There were no

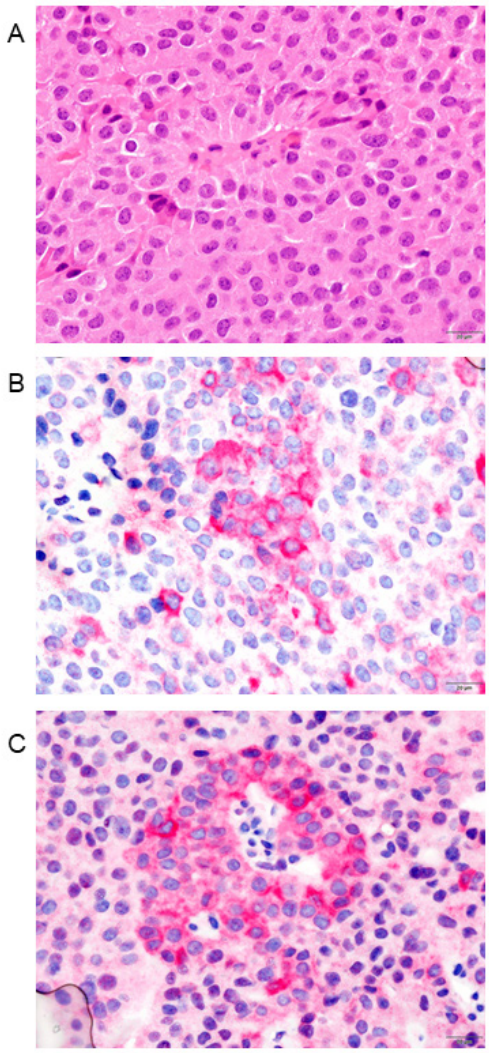

Figure 2. Permanent pathology reveals a gonadotroph adenoma (A; hematoxylin and eosin stain; H&E×60), and immunohistology reveals luteinizing hormone (B; ×60) / follicle-stimulating hormone (C; ×60) -positive intracellular staining.

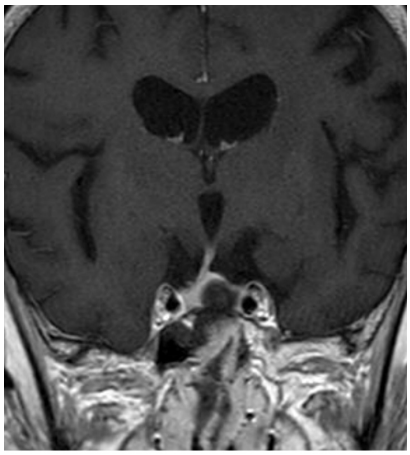

Figure 3. Postoperative gadolinium-T1 magnetic resonance imaging shows no tumor.

complications, such as cerebrospinal fluid leakage or diabetes insipidus. The patient's hyponatremia, cognitive decline, movement retardation, loss of appetite, dressing apraxia, and edema of the limbs markedly improved. The patient was prescribed hydrocortisone (15 mg/day) and was discharged. Two months postoperatively, he underwent an endocrine tolerance test. The CRH loading test showed normal reactivity of ACTH and reduced reactivity of cortisol. The TRH loading test showed normal reactivity of TSH. The LH-RH loading tests showed reduced reactivities of LH/FSH, while the baseline levels of LH/FSH had improved to the normal levels compared to the preoperative values. All other test results were normal. No recurrence was observed within the first 14 postoperative months, and the ACTH/cortisol secretion abilities were found to have improved; the oral administration of hydrocortisone was gradually discontinued. At 30 months after eTSS, he was being followed as an outpatient with modified Rankin Scale score 0.

3. Discussion

This may first appear as a case where a pituitary tumor was accidentally discovered by close examination of cognitive symptoms, but the principal symptoms were caused by hormonal disorder; therefore, the case does not fit the definition of pituitary incidentaloma (*2-4*). Aszalós (*5*) reported that the connection between the central nervous system and the endocrine system is extremely complex. The hypothalamus serves as a crucial center for the integration and coordination of autonomic functions by neuronal and hormonal pathways. It plays a central role in the homeostatic regulation of internal physiological conditions. It controls growth and reproduction, stress reactions, and determines rhythmicity, periodicity, and timing of physiological processes. CRH acts as a neurotransmitter; it has a special role in stress-behavior, anxiety, and depression and it blocks deep sleep. The most characteristic neurological sign of PA is the visual field defect. The main psychiatric symptom of hypopituitarism is a combination of dementia and delirium (*5*).

Benvenga (*6*) *et al*. reported that central hypothyroidism (CH) is a rare cause of hypothyroidism. CH is frequently overlooked, as its clinical picture is subtle and includes non-specific symptoms; furthermore, if measurement of TSH alone is used to screen for thyroid function, TSH concentrations can be normal or even above the upper normal reference limit. Indeed, certain patients are at risk of developing CH, such as those with a pituitary adenoma or hypophysitis, those who have been treated for a childhood malignancy, have suffered a head trauma, sub-arachnoid hemorrhage or meningitis, and those who are using drugs capable of reducing TSH secretion (*6*). Adult-onset CH, as is the case in primary hypothyroidism, involves symptoms such as lethargy, fatigue, eyelid edema, feeling cold, weight gain, bradykinesia, lethargy, memory loss, constipation, and crying. However, caution is required because these symptoms may be suppressed by other hormone-producing tumor symptoms or symptoms of anterior pituitary hormone secretion deficiency. Particularly when the ACTH/cortisol functions are disordered, hypofunction-associated consciousness disturbance may ensue; hence, it is challenging to distinguish the symptoms of hypothyroidism (*7*). In addition, hypogonadism, GH deficiency, and diabetes insipidus may be recognized simultaneously, which may render diagnosis difficult.

In our case, the patient was diagnosed with CH and secondary hypoadrenocorticism, caused by an otherwise normal pituitary being compressed by the tumor, based on the clinical course, imaging findings, and endocrinological examination. A PubMed search showed that this is the first diagnosed case of a gonadotroph adenoma mimicking dementia. Early diagnosis and treatment of this case resulted in complete remission, and we plan to follow the patient in the long term.

In conclusion, we reported the rare case of a patient with a gonadotroph adenoma mimicking dementia. Secondary hypothyroidism and secondary hypoadrenocorticism due to tumor, primarily caused the condition. It is important to consider PA in the differential diagnosis of dementia, and early diagnosis and treatment can contribute to a patient's good clinical outcome.

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Letter

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Successful percutaneous computed tomography guided drainage of mediastinal abscess in esophageal perforation

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Summary **Esophageal perforation with subsequent development of a mediastinal abscess is a well-known clinical entity. Etiologies including idiopathic and iatrogenic with invasive procedures have been reported in medical literatures. This condition is seriously associated with high co-morbidity and in some cases especially if intervention has not been applied associated with high mortality. For long time, open surgical intervention was the only available treatment modality for esophageal perforation with subsequent development of a mediastinal abscess. However, recently there are some other less invasive modalities that have been used with comparable if not preferable success including; self-expandable metallic or plastic stents and imaging guided percutaneous drainage of the mediastinal abscess combined with stenting. We report a patient who presented with esophageal perforation complicated with a mediastinal abscess that was treated successfully with an imaging guided percutaneous drainage of the mediastinal abscess. This case is to emphasize on the fact that endoscopic stent placement is safe and effective for esophageal perforations. Percutaneous CT-guided drainage of associated mediastinal abscesses is an uncommon procedure, but the results suggest that it is associated with high technical and clinical success rates. There should be increased involvement of interventional radiology in the management of those cases.**

Keywords: Esophageal perforation, mediastinal abscess, CT guided drainage

Esophageal perforation is a rare but potentially fatal surgical emergency with high mortality rates. Factors contributing to high morbidity and a mortality rate of >20% include proximity of vital organs, difficulty to access the esophagus, the lack of a strong serosa and complex blood supply of itself (*1-3*). Perforation can be either idiopathic or iatrogenic which is commonly a result of endoscopic procedures (esophagectomy, emetogenic, or food impaction). Spontaneous rupture without pre-existing pathology of the esophagus has been reported in about 15% of the cases (*4*).

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A 45-year-old male presented to the emergency department with sudden onset of throat spasms, and dysphagia after accidental ingestion of a bone fragment while eating a T-bone steak. Initial vitals showed sinus tachycardia and significant distress on physical exam. A computed tomography (CT) of chest showed extravasation of contrast into the mediastinum and pneumomediastinum. Esophagogram showed esophageal perforation and leak 10cm proximal to gastroesophageal (GE) junction. Endoscopy was performed, bone was retrieved with placement of covered esophageal stent. It showed a non-bleeding perforation with adjacent mucosal erythema. On day-7, patient had high fevers with up-trending white blood cell count (WBC: 25 k/uL). CT chest showed pneumomediastinum with periesophageal abscess extending from carina to GE junction surrounding stent (Figure 1A and 1B).

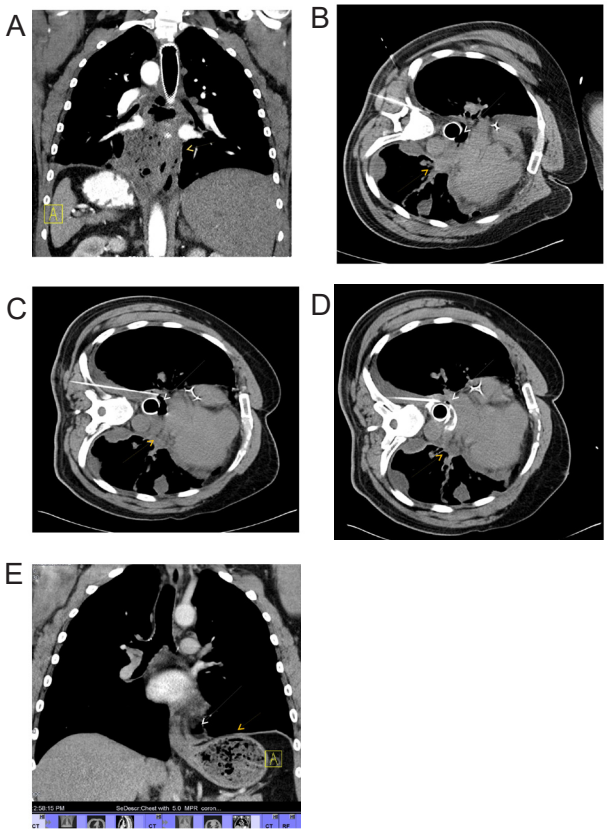

Figure 1. Computed tomography of chest showing (A) pneumomediastinum with periesophageal abscess extending from carina to GE junction (Yellow arrow), (B) pneumomediastinum (white arrow) with periesophageal abscess (yellow arrow), (C) advancement of pigtail catheter into periesophageal abscess, (D) advancement and dilatation of pigtail catheter into periesophageal and mediastinal abscess, and (E) complete resolution of abscess.

At that point interventional radiology performed CT-guided drainage of mediastinal abscess with seldinger-technique and placement of a left posterior suction drain. A 25-gauge Chiba needle was advanced into the collection with CT guidance. With the needle tip in the collection, the inner trocar needle was removed, and a fluid sample was obtained for microbiologic analysis. Thereafter, a guide wire was advanced into the abscess. The percutaneous tract was then serially dilated with serial dilators, after which a locking pigtail catheter was then advanced over the guide wire and into the collection (Figure 1C and 1D). At the time of initial drainage, all purulent material was aspirated to completion and flushed with 5-10 mL sterile saline solution to ensure complete drainage. The catheters were then secured to the skin with a retention device and placed to gravity drainage. Following drainage, WBC trended down, followed by complete resolution of abscess (Figure 1E). Stent was removed followed by complete recovery.

The principles of management of esophageal perforation include broad spectrum IV antibiotics, gastric decompression, control of ongoing leak, surgical debridement of infected tissue and drainage of mediastinal cavity especially with acute septicemia. Operative repair for esophageal perforation has been gold standard treatment for an acute perforation, however, paradigm shift towards the use of self-expandable metallic and plastic stents has been seen especially in the absence of acute septicemia and with limited mediastinal or pleural contamination. Complications of self-expandable stents include stent migration, leakage, stent perforation or bleeding. Mediastinal abscess is one of the leading causes of mortality after esophageal perforation which requires adequate drainage (*5*). Imaging guided percutaneous drainage of the mediastinal abscess combined with stenting is an emerging alternative treatment to open drainage with potentially high technical and clinical success. In 2011 Arellano *et al*., conducted a trial in patients with mediastinal abscess who underwent CT guided drainage (n = 23) over a period of 10 years. Out of 23 patients, 22 patients had complete resolution of abscess and they didn't require any surgical intervention with a success rate of 95.6% (*6*).

In our patient successful drainage was performed with less invasive seldinger-technique, with the patient scanned in right-lateral-decubitus. Patient positioning was determined based on the shortest and safest route to the abscess. Images were then analyzed to determine the safest percutaneous route to the abscess that avoided the esophagus, the lung, or the internal mammary or intercostal arteries. Despite adequate safety and efficacy of stents in acute esophageal perforation, a head to head trial with surgical repair is still lacking.

Ben-David K *et al*., conducted a retrospective review of acute esophageal perforation cases from 2007 through 2013 (n = 76). All patients were treated within first 24 hours of presentation with a removable covered esophageal stent. Median length of stay at ICU and hospital was 3 and 10 days respectively with median hospital charges of $85,945. The mortality rate was 1.3% in first 30 days (*7*). Likewise, Richard K freeman *et al*., evaluated 60 patients who had either stent placement or surgical intervention for iatrogenic esophageal perforation from 2009 through 2012. They compared the cost and the outcomes (mortality, morbidity, length of hospital stay) within 2 cohorts. Their results showed both modalities of treatment were equally effective, however, stent placement was seen to be more cost effective with less mortality and morbidity rates. They noticed a significant difference in morbidity (17% *vs*. 43%, p = 0.02), mean length of hospital stay (6 *vs*. 11 days, p = 0.0007), time for oral intake (3 *vs*. 8 days, p = 0.0004), and cost ($91,000 *vs*. 142,000, $p < 0.0001$) in patients who had stent placement in comparison to the ones getting surgical repair (*8*).

Further head to head clinical trials are required to compare esophageal stents and CT guided drainage of mediastinal abscess with open surgical repair to reduce overall morbidity, mortality and cost of hospital.

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Letter

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Focusing on basic data and a model of healthcare security for rare diseases: The Multidisciplinary Expert Seminar on Healthcare Security for Rare Diseases in China was held in Beijing

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Summary **On August 10, 2019, the Multidisciplinary Expert Seminar on Healthcare Security for Rare Diseases in China was held in Beijing. The seminar was organized by the Shanghai Foundation for Rare Disease and Shanghai Health Development Research Center and advised by the China Alliance of Rare Diseases. Participants in this seminar included government officials, experts in clinical medicine, pharmacy, epidemiology, health economics, and law as well as representatives from rare disease patient organizations. The participating experts cited three key elements of healthcare security, including its concept, data, and mechanism, to solve the problem of health care security for patients with rare diseases at the national level. Collection of basic data and creation of a model of healthcare security for rare diseases were discussed. Data collection should be actively promoted. Creation of a special zone to ensure medical care for patients with rare diseases should be considered. Healthcare security should be classified, which means that basic medical insurance provides better care for rare diseases that respond to treatment, and channels should be established for rare diseases that respond poorly to treatment.**

Keywords: Rare disease, healthcare security, multidiscipline, expert discussion

On August 10, 2019, the Multidisciplinary Expert Seminar on Healthcare Security for Rare Diseases in China was held in Beijing. The seminar was advised by the China Alliance of Rare Diseases (CARD) and organized by the Shanghai Foundation for Rare Disease and Shanghai Health Development Research Center. Present at the meeting, were relevant officials from the Department of Drug Policy and Essential Medicine System of the National Health Commission (NHC), the Bureau of Medical Administration of NHC, and members from the Expert Committee on Diagnosis and Treatment of and Care for Rare Diseases of the NHC, experts and scholars from rare disease societies in 14 provinces or municipalities, medical insurance associations, and the fields of clinical medicine, pharmacy, epidemiology, health economics, and law, as well as representatives from rare disease patient organizations. In total, more than 80 experts attended the seminar.

Wenjiong He, vice president of the China Association of Social Security and professor in the School of Public Administration of Zhejiang University, gave a special speech on "Examination and Implementation of Healthcare Security for Rare Diseases in China." His research indicated that medical care for rare diseases in China is expensive, and this burden is mainly borne by patients and their families. He also described healthcare security for rare diseases in Qingdao, Shanghai, and Zhejiang. He interpreted the latest policy of the National Healthcare Security Administration, which calls for the issuance of new policies to be halted in order to expand the scope and increase the level of reimbursement by basic medical insurance at the district level and which proposed a list of ensured medical benefits (*1*). He pointed out that the introduction of this policy meant that issuing new local policies on ensured medical care for rare diseases

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would be difficult. Therefore, the problem of ensuring medical care for rare diseases needs to be gradually and effectively solved at the national level. The key lies in three aspects: the concept of ensured care for rare diseases, basic data on patients with rare diseases, and a mechanism to ensure care for rare diseases.

Junshuai Liu, vice president of the Qingdao Medical Insurance Research Association of Shandong Province, gave a speech entitled "Some Thoughts on Ensuring Medical Care for Rare Diseases in China." He described the basic aspects of ensured medical care for rare diseases (15 aspects including financing, coordination, cataloguing, pricing, and reimbursement) along with healthcare in Qingdao, and he analyzed its relevant points. He pointed out that healthcare security for rare diseases is a condensed version and a touchstone of healthcare reform in China. He recommended that healthcare security for rare diseases be a special zone for reform of healthcare security. The healthcare security should be independently funded. He also pointed out that in the event of a large surplus in national medical insurance (up to 2.3 trillion RMB in 2018) (*2*), allocating part of that surplus would ensure care for patients with rare diseases.

A spirited discussion on ensuring medical care for rare diseases in China was held among participants. The discussion specifically mentioned basic data collection and creation of a model of healthcare security for rare diseases in China, and participants actively made suggestions.

The participating experts fully recognized the significance of basic data to devising policies to ensure medical care for rare diseases in China. Linkang Li, chairman of CARD, mentioned that surveys on the three aspects of healthcare security were currently underway to obtain data and facilitate decision-making, including a health economics evaluation of rare diseases, a survey of doctors' attitudes towards the cooperative network for diagnosis and treatment of rare diseases, and a sociological study of patients with rare diseases through patient organizations. Shuyang Zhang, vice chairman and secretary general of CARD, mentioned that more than 38,000 patients with rare diseases have been registered since December 2016 through precision medicine research plans and cohort studies of rare diseases (*3*). She then appealed for more support from participating institutions to register patients. Ruilin Song, vice chairman of CARD, suggested that a mandatory rare disease reporting system should be established as soon as possible. Yuhui Zhang, deputy director of the National Health and Development Research Center of the NHC, proposed that patients with rare diseases be screened from the health expenditure database and that related research be conducted.

Experts at the meeting had a lively discussion of the model to healthcare security for rare diseases in China. A considerable number of the experts supported creation of a special zone to ensure medical care for rare diseases, a point raised by Junshuai Liu. However, some experts suggested that this may lead to inequity. In addition, some experts suggested increasing the amount of maternity insurance financing to ensure care for patients with rare diseases. Feng Zhang, deputy director of the Department of Drug Policy and Essential Medicine System of the NHC, pointed out that a multi-faceted comprehensive system of healthcare security for rare diseases is required and that it should be tailored to different diseases and areas. Specifically, medical care should be classified based on the response to treatment and cost of rare diseases (basic medical insurance provides better care for rare diseases that respond to treatment, and channels should be established to ensure care for rare diseases that respond poorly to treatment). Differences in policies in different regions should be allowed because of the various socio-economic levels in China.

After the seminar, the Expert Committee on Diagnosis and Treatment of and Care for Rare Diseases of the NHC and CARD held a symposium on the inclusion principle of China's Second List of Rare Diseases.

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