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Review

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Epidemiology and distribution of 207 rare diseases in China: A systematic literature review

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SUMMARY Epidemiological data on rare diseases in China are currently limited. The objective of this study was to provide a comprehensive understanding of the prevalence and incidence of rare diseases by systematically analyzing the available epidemiological data. We conducted a comprehensive search of English and Chinese databases, the Incidence and Prevalence Database, the Chinese Rare Disease Guideline, and the Taiwan Health Promotion Administration from 2010 to 2023. We identified the top diseases and regions based on epidemiological data and present the maximum, minimum, and median prevalence and incidence values in tables and forest plots. 1,264 prevalence and incidence data were retrieved from 277 studies, guidelines and official websites, covering 110 rare diseases (53.1%) and 32 regions (94.1%). In terms of geographical regions, incidence or prevalence data were available for 32 regions (94.1%), excluding Tibet Hui Autonomous Region and Macao Special Administrative Region. In terms of rate, 60 and 77 out of 207 diseases (29.0% and 37.2%) had available incidence and prevalence data, respectively. Eight diseases had an incidence rate equal to or greater than that of 1,000 patients per million. The present study provides a comprehensive epidemiological analysis and valuable insights into the prevalence and incidence of rare diseases in China. Our findings underscore the pressing need for sustained drug research and medical support for individuals and families impacted by rare diseases.

Keywords rare disease, epidemiology, China

1. Introduction

Rare diseases (RDs) are a class of diseases characterized by a low incidence, low prevalence, and low total number of patients. RDs encompass a wide range of diseases that exhibit distinct features, such as variable types, inheritance patterns, difficult diagnoses, severe conditions, and low treatability (*1*). Globally, there are more than 7,000 known RDs, with more than 250 million patients affected (*2*). In China, 207 diseases are listed as RDs by the government, affecting an estimated 20 million patients (*3*). RDs place a significant economic burden on patients and their families (*4*). In recent years, the Chinese government has given increasing attention to RDs with the rapid development of the country (*5*). The establishment and release of the National Rare Diseases Registry System of China (NRDRS) and the first batch of RD catalogs are significant milestones toward improving the diagnosis, treatment, and protection of patients with RDs in China (*6-8*). Furthermore, the number of RD drugs covered by medical insurance in China continues to increase, providing patients with greater access to necessary medical care, ultimately leading to better health outcomes and quality of life (*5,6,9*).

The current epidemiological research on RDs in China is insufficient (*1,10,11*). The absence of comprehensive epidemiological data, the availability of scattered and incomplete data, and a lack of systematic retrospective epidemiological analyses are notable issues. To address these shortcomings, this study aimed to systematically summarize the incidence, prevalence and corresponding population information of RDs in China by conducting a thorough search of variable sources, including academic literature on the epidemiology of RDs in China, RD guidelines, and official RD information websites. Through this comprehensive analysis, we aimed to gain a comprehensive understanding of the epidemiology and distribution of RDs in China. Thorough research on RDs requires relevant epidemiological studies as the basis. This study not only provides the basis for basic medicine, clinical medicine, and economic and social research related to RDs but also has the potential to inform the development of a standard consensus on the definition of RDs in this field and has significant implications for

evaluating the RD drug market.

We present this article in accordance with the PRISMA reporting checklist.

2. Materials and Methods

The search process in this study adhered strictly to the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) guidelines (*12*).

2.1. Search strategy

The relevant literature published between 1 January 2010 and 31 December 2023 in both English and Chinese databases was identified. The English databases included PubMed and Web of Science. The Chinese databases included SinoMed, China National Knowledge Internet (CNKI), Wangfang database, Vip database. The search strategy utilized a general search string was: disease name [Title/Abstract/Text Word] AND (prevalence [Title/Abstract/Text Word] OR incidence [Title/Abstract/Text Word]). Additionally, we supplemented our search with data from Incidence and Prevalence Database® (IPD), as well as from Guidelines for Diagnosis and Treatment of Rare Diseases (2019 edition) and the Taiwan Health Promotion Administration. This approach was taken to avoid missing relevant epidemiological data. The full details of the search strings are provided in Supplemental Table S1 (*http://www.irdrjournal.com/action/getSupplementalData.php?ID=195*).

2.2. Eligibility criteria

Reviews, letters to the editor, case reports, case studies, original research literature, academic dissertations and conference abstracts were included. The inclusion and exclusion criteria are shown in Figure 1.

2.3. Data extraction

The data were extracted using Microsoft Excel 2019. The target data were categorized into three parts: basic epidemiological data, additional data and notes. *i*) Basic epidemiological data included the disease name, the available ICD-10 codes, the subcategory of disease, the incidence and prevalence, and the region, age, sex, and ethnicity of the patients with corresponding epidemiological data. *ii*) Additional data included the number of participants, the observation period and the year of publication. *iii*) Notes included the data remarks (whether the data were standardized), literature remarks (whether the study was a retrospective analysis, systematic review, meta-analysis or original research), and citations of the literature, literature links and data sources (literature or guidelines). The data were retrieved from the aforementioned sources by two team members. The results were subsequently summarized on a single table using the "entry tested twice" approach to ensure accuracy. Finally, a third member reviewed the table for further verification.

2.4. Statistical analysis

Forest plots were generated based on the maximum and minimum rates of the disease. However, if there were at least three data points available and all of them were precise values rather than ranges, the median value was also included in the plot. All the forest plots were generated using GraphPad Prism software, version 8.0 (San Diego, California, USA).

The search strategy summary can be seen in Table 1.

3. Results

A total of 1,264 incidence and prevalence data points

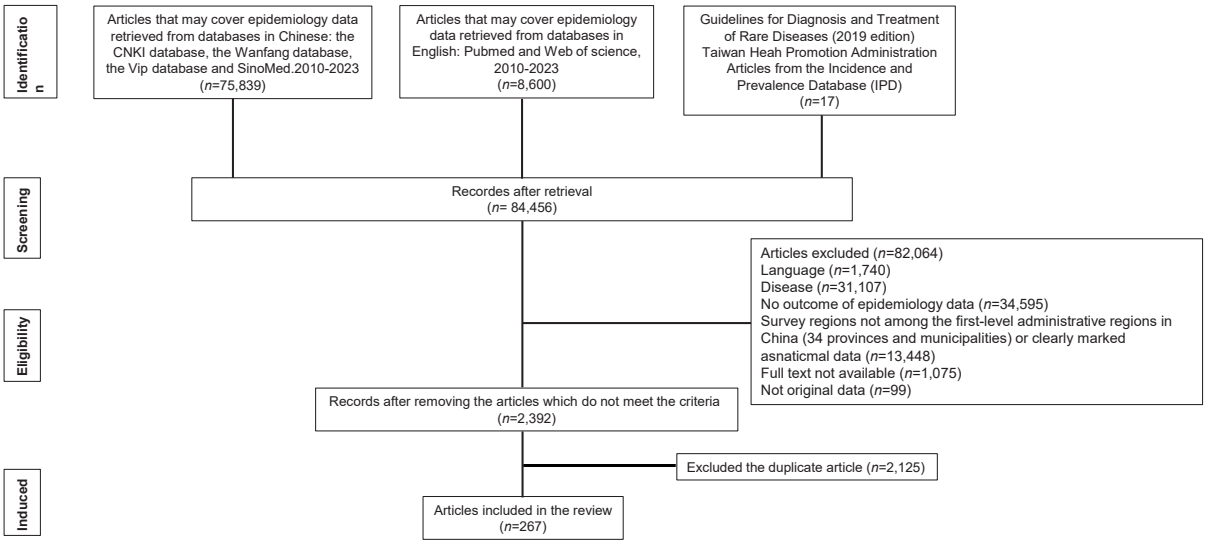

Figure 1. Study flow diagram. Abbreviations: CNKI, China National Knowledge Infrastructure.

Table 1. The search strategy summary

Items	Specification
Date of search (specified to date, month and year)	From 1 January 2010 to 31 December 2023.
Databases and other sources searched	PubMed, Web of Science, SinoMed, China National Knowledge Internet (CNKI), Wangfang database, Vip database, Incidence and Prevalence Database® (IPD), the Chinese Rare Disease Guideline (2019) and the Taiwan Health Promotion Administration.
Search terms used (including MeSH and free text search terms and filters)	Please see Supplementary Table S1.
Timeframe	Last retrieval on 11 February 2024.
Inclusion and exclusion criteria (study type, language restrictions, *etc.*)	Inclusion criteria: the literature may cover epidemiology data retrieved from databases above which published from 2010 to 2023. Exclusion criteria: the literature without outcome of epidemiology data, survey regions among the 34 regions original data.
Selection process (who conducted the selection, whether it was conducted independently, how consensus was obtained, *etc.*)	The data were extracted using Microsoft Excel 2019. The data were retrieved from the aforementioned sources by two team members. The results were subsequently summarized on a single table using the "entry tested twice" approach to ensure accuracy. Finally, a third member reviewed the table for further verification.

were extracted from 277 literature sources that met the retrieval requirements (Table 2, online data, *http://www.irdrjournal.com/action/getSupplementalData.php?ID=195*).

Among the diseases included in the study, 110 of the 207 diseases examined (53.1%) had available incidence or prevalence data. The five diseases with the largest number of cumulative incidence and prevalence data points were Phenylketonuria (374 data points), Melanoma (220 data points), Multiple Sclerosis (123 data points), Hepatolenticular Degeneration (Wilson Disease) (43 data points) and Malignant pleural mesothelioma (30 data points).

In terms of geographical regions, incidence or prevalence data were available for 32 regions (94.1%), excluding Tibet Hui Autonomous Region and Macao Special Administrative Region. The five regions with the highest number of cumulative incidence and prevalence data points were Taiwan Province (143 data points), Zhejiang Province (83 data points), Inner Mongolia Autonomous Region (81 data points), Liaoning Province (62 data points), and Qinghai Province (58 data points). On the other hand, the five regions with the lowest number of cumulative incidence and prevalence data points were Tibet Hui Autonomous Region (zero data point), Macao Special Administrative Region (zero data point), Hubei Province (one data points), Jiangsu Province (two data points), and Heilongjiang Province (three data points).

According to the 1,264 incidence and prevalence data, 502 (39.7%) were related to the newborn population. Among the 207 diseases considered, 36 were classified as an Inherited Metabolic Disease (IMD) (*27*), and only four out of these 36 IMDs (Hereditary Hypomagnesemia, Hyperornithinaemia-Hyperammonaemia-Homocitrullinuria, *N*-acetylglutamate Synthase Deficiency, Very Long Chain Acyl-CoA Dehydrogenase Deficiency and X-linked Agammaglobulinemia) lacked any available incidence or prevalence data (Table 3).

3.1. Incidence

A total of 921 incidence data points were extracted from 277 literature sources that met the retrieval requirements.

Regarding the scope of the study, 60 out of 207 diseases (29.0%) had available incidence data. The five diseases with the highest number of reported incidence data were Phenylketonuria (372 data points), Gastrointestinal stromal tumor (35 data points), Malignant pleural mesothelioma (30 data points), Hepatolenticular Degeneration (Wilson Disease) (24 data points), and Methylmalonic Academia (nineteen data points).

Regarding geographical regions, incidence data were available for 32 out of 34 regions (94.1%). The five regions with the largest number of reported incidence data points were Taiwan Province (59 data points), Beijing (43 data points), Inner Mongolia Autonomous Region (36 data points), Shanghai (31 data points) and Xinjiang Uygur Autonomous Region (24 data points). The five regions with the lowest number of cumulative incidence data points were Tibet Hui Autonomous Region (zero data point), Macao Special Administrative Region (zero data point), Hubei Province (one data points), Jiangsu Province (two data points), and Heilongjiang Province (three data points).

Additionally, there were eight diseases with an incidence equal to or greater than 1,000 patients per million (ppm): Homocysteinemia (275,000 ppm), Retinopathy of prematurity (179,000), Retinoblastoma (2,121 ppm), Non-Syndromic Deafness (1,860 ppm), Phenylketonuria (1,480 ppm), Isovaleric Acidemia

Table 3. Lists of inherited metabolic diseases among the 121 rare diseases

Disease Name	Number of incidence data point	Number of prevalence data point
21-Hydroxylase Deficiency	3	0
Arginase Deficiency	1	0
Beta-ketothiolase Deficiency	7	0
Biotinidase Deficiency	0	1
Carnitine Deficiency	10	6
Citrullinemia	5	2
Galactosemia	4	2
Glutaric Acidemia Type I	4	2
Hereditary Fructose Intolerance	1	0
Hereditary Hypomagnesemia	0	0
Holocarboxylase Synthetase Deficiency	1	1
Homocysteinemia	2	13
Homozygous Hypercholesterolemia	0	1
Hyperornithinaemia-Hyperammonaemia-Homocitrullinuria Syndrome	0	0
Hyperphenylalaninemia	1	0
Hypophosphatasia	1	2
Isovaleric Acidemia	8	1
Long Chain 3-hydroxyacyl-CoA Dehydrogenase Deficiency	1	0
Lysinuric Protein Intolerance	1	0
Lysosomal Acid Lipase Deficiency	0	1
Maple Syrup Urine Disease	7	4
Medium Chain Acyl-CoA Dehydrogenase Deficiency	9	8
Methylmalonic Academia	13	7
Multiple Acyl-CoA Dehydrogenase Deficiency	2	0
N-acetylglutamate Synthase Deficiency	0	0
Neonatal Diabetes Mellitus	0	1
Ornithine Transcarbamylase Deficiency	2	0
Phenylketonuria	329	0
Porphyria	0	1
Progressive Familial Intrahepatic Cholestasis	0	1
Propionic Acidemia	0	2
Sitosterolemia	0	1
Tetrahydrobiopterin Deficiency	1	1
Tyrosinemia	0	2
Very Long Chain Acyl-CoA Dehydrogenase Deficiency	0	0
X-linked Agammaglobulinemia	0	0

(1,207.6 ppm), Maple Syrup Urine Disease (1,107.0 ppm), and Noonan Syndrome (1,000 ppm).

The five diseases with the widest range of reported incidence values were Homocysteinemia (range: 274,996.7 ppm), Retinopathy of prematurity (range: 86,160 ppm), Retinoblastoma (range: 2,120.3 ppm), Phenylketonuria (range: 1,471 ppm) and Isovaleric Acidemia (range: 1,207.0 ppm) (Figure 2A).

3.2. Prevalence

A total of 343 prevalence data points were extracted from 277 literature sources that met the retrieval requirements.

Within the scope of the study, 77 out of 207 diseases (53.7%) had available prevalence data. The five diseases with the highest number of reported prevalence data points were Multiple Sclerosis (87 data points), Hepatolenticular Degeneration (Wilson Disease) (24 data points), Hemophilia (24 data points), Hidradenitis suppurativa (fifteen data points), Homocysteinemia (thirteen data points) and Methylmalonic Academia (eight data points).

Regarding geographical regions, prevalence data were available for nineteen out of 34 regions (55.9%). The five regions with the largest number of reported prevalence data points were Taiwan Province (69 data points), Zhejiang Province (58 data points), Liaoning Province (47 data points), Guangdong Province (47 data points), and Inner Mongolia Autonomous Region (46 data points). On the other hand, there were 15 provinces, autonomous regions and municipalities (44.1%) that lacked any reported prevalence data. These locations were Tibet Autonomous Region, Macao Special Administrative Region, Shanxi Province, Jilin Province, Heilongjiang Province, Jiangsu Province, Hubei Province, Sichuan Province, Guizhou Province, Yunnan Province, Shaanxi Province, Gansu Province, Ningxia Hui Autonomous Region, Xinjiang Uygur Autonomous Region, and Hainan Province.

Additionally, the five diseases with the highest reported prevalence rates were Homocysteinemia (743,962.5 ppm), Retinopathy of prematurity (128,000 ppm), Primary biliary cholangitis (4,150.5 ppm), Hidradenitis suppurativa (1,856 ppm) and Fragile X syndrome (800 ppm).

The five diseases with the widest range of reported

prevalence values were Homocysteinemia (range: 5,825,962.5 ppm), Primary biliary cholangitis (3,980.5 ppm), Hidradenitis suppurativa (range: 1,750 ppm), Phenylketonuria (range: 281.6 ppm) and Congenital Hyperinsulinemic Hypoglycemia (range: 229.1 ppm) (Figure 2B).

4. Discussion

4.1. Basic epidemiological information

This study examined 207 RDs and found that 97 (46.9 %) lacked data on disease incidence and prevalence. Among the 110 diseases with available incidence or prevalence

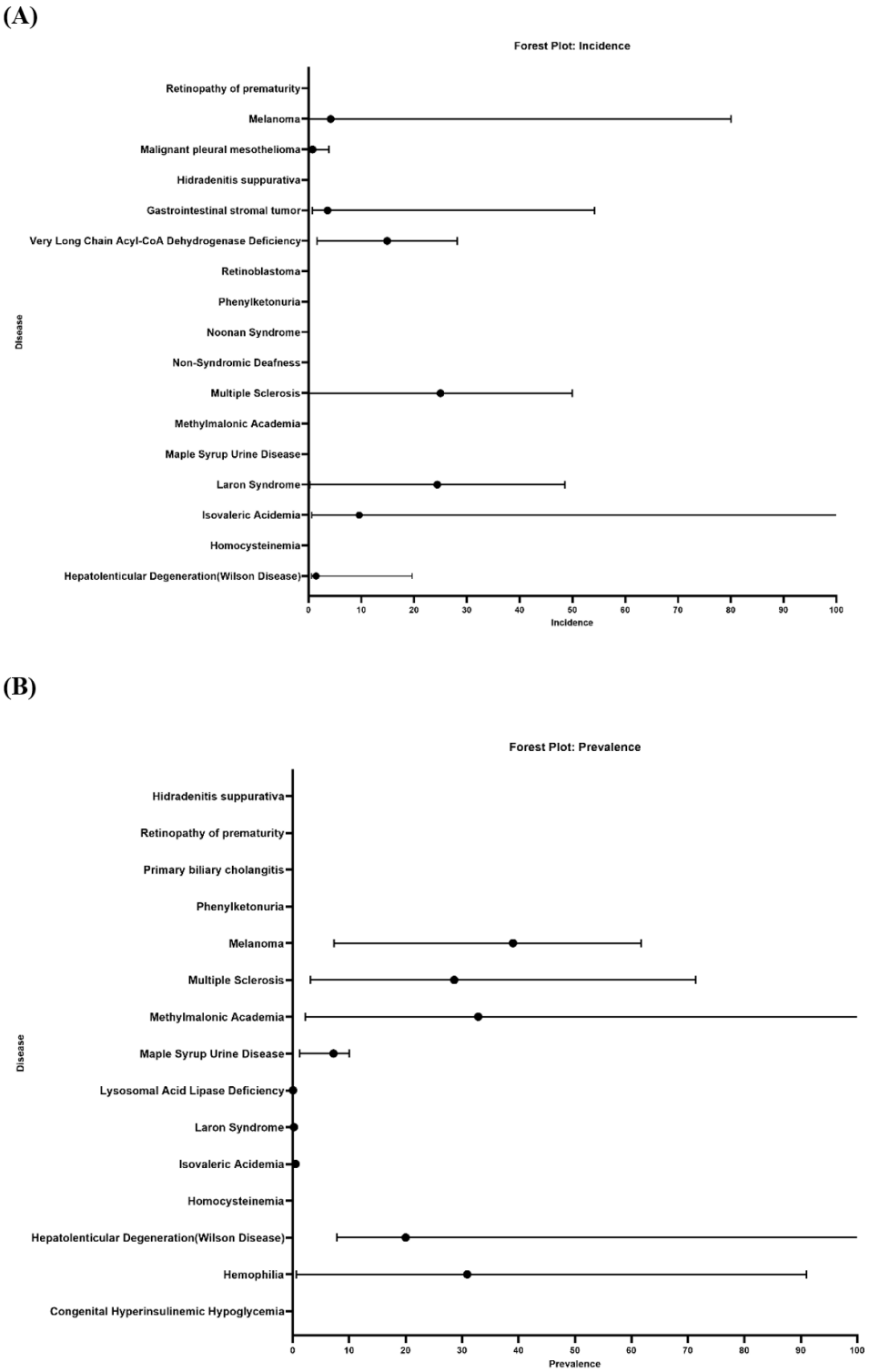

Figure 2. Incidence and prevalence rates of RDs. (A) Incidence rates of RDs; **(B)** Prevalence rates of RDs. Abbreviations: RDs, rare diseases; PPM, patients per million. *Notes:* The black points represent the medians, and the two ends of the line represent the extreme values. A line is displayed when the amount of data is no less than 3 and the data are not approximately within the range. The data were not available if there are no data for the disease.

data, 38 had only one prevalence value, and 14 had only one incidence value. These findings indicate a significant amount of missing disease and regional epidemiological data. As we can see from the results, there are currently several challenges in the epidemiological study of RDs in China. Although the Chinese government has released the two RD catalogs, there is still no universally recognized standard for defining which is RDs (*280*). This lack of standardization could be attributed to two main factors: insufficient epidemiological information on RDs and heterogeneity in the existing incidence and prevalence data (*281,282*).

4.2. The reasons for insufficient epidemiological information and heterogeneity

The reasons for the lack of epidemiological data on RDs are as follows. First, there are few patients of RDs, resulting in insufficient research for this topic. Second, RDs are difficult to diagnose (*62*). Third, the RD management system (NRDRS) in China has not yet been covered nationwide (*5*). Statistics concerning patient household registration and place of medical treatment are incomplete, increasing the regional bias caused by remote medical treatment (*5,11*).

Furthermore, substantial heterogeneity in the incidence or prevalence of the same disease was observed across different regions or populations. For instance, eight diseases (6.6%) had an incidence or prevalence rate exceeding 1,000 ppm. The disease with the highest incidence rate was Homocysteinemia (2-390,800 ppm), and this disease also had the highest prevalence rate Homocysteinemia (37.5-744,000 ppm). Moreover, heterogeneity was not limited to different diseases but was evident within the same disease. For example, Lysosomal Acid Lipase Deficiency had a prevalence of 0.04 ppm, which is lower than that of Homocysteinemia. Similarly, incidence of Laron Syndrome was 0.2 ppm, which was lower than the maximum incidence of Homocysteinemia. Heterogeneity exists in terms of epidemiological rates and data volume in different regions due to differences in prevalence and allopathic treatment (*283*). The findings show that the greater the level of social development is, the greater the proportion of patients treated, resulting in a lower rate than that reported in previous studies (*283*). These variations further complicate the establishment of a unified definition standard for RDs.

To address these challenges and enhance academic quality, this study systematically collected and summarized incidence and prevalence data, along with corresponding population information such as region, sex, age, and race. By consolidating scattered epidemiological information, researchers can more easily access the essential epidemiological details of RDs. This comprehensive analysis also aids researchers in identifying RDs with high heterogeneity in incidence or prevalence rates. Furthermore, by considering the population-specific characteristics associated with the data, this study provides valuable insights into the number of patients affected and serves as a reference for reaching a consensus on the definitions of RDs through epidemiological data.

4.3. Research and development of RD drugs

Although significant progress has been made in the development of RD drugs in China, there are still diseases for which there is no reference indication for drug treatment. Currently, based on data from the Pharnexcloud (*https://data.pharnexcloud.com/*, a website providing Chinese drug information) and our research findings, out of the 110 diseases with available incidence or prevalence data, only 43 (39.1%) have drugs with reference indications. In contrast, among the 97 diseases without any incidence or prevalence data, only fifteen (15.5%) have drugs with reference indications. This finding suggested a positive correlation between the completeness of epidemiological information for RDs and the availability of corresponding drugs (*284*).

This phenomenon can be attributed to several factors. First, the acquisition of epidemiological data serves as the initial step in the research and development of drugs. For RDs without any available drugs, the lack of systematic epidemiological data prevents pharmaceutical companies from adequately evaluating market demand and potential profitability. Consequently, limited commercial viability restricts the investment and production capabilities of pharmaceutical companies in the field of RDs (*5*).

Second, comprehensive epidemiological studies in terms of efficacy play a crucial role in identifying a larger patient population. Only through epidemiological studies on patient cohorts can the effectiveness of drugs be determined, thus facilitating the transition of new drugs from clinical trials to the market (*285*). This emphasizes the importance of regular epidemiological investigations and summaries of the development of RD drugs. Given that there are still 95 RDs in China without any drugs with reference indications, this study collected epidemiological information for 85 diseases, thereby providing assistance for the development of drugs for at least 53 RDs. From the perspective of benefiting patients with RDs, the introduction of new drugs enables the possibility of treatment.

However, the key to alleviating the economic burden on these patients lies in the inclusion of RD drugs in medical insurance coverage. In 2019, Zhejiang Province became the first region in China to establish a provincial drug security system for RDs (*286*). The inclusion criterion for RD drugs in medical insurance was epidemiological information obtained from the neonatal

disease screening information system. It is worth noting that among the 112 drugs for which RD was used as a reference indication, 57 (50.9%) are still not covered by medical insurance, indicating room for improvement in the inclusion rate of RD drugs in medical insurance schemes. By collecting epidemiological information on 207 RDs nationwide, this study provides a valuable reference for the inclusion of RD drugs in medical insurance. He *et al*. noted that if RDs identified can be included only in medical insurance, it will strongly promote patients to apply for certification and doctors to report diseases (*10*). Furthermore, it offers support for disease identification, aiding in the expansion of regional and disease-specific RD medical security systems (*287*).

4.4. Future challenges and opportunities of RDs in China

However, there are still limitations in our research. The amount of epidemiological data is currently limited, and the data cannot be critically analyzed due to the late start of RD research in China (*1,10,11*). However, the findings of this study significantly contribute to filling the gaps in epidemiological information concerning RDs in China. These gaps include 52 diseases with only one reported incidence or prevalence value within a single region, as well as 106 RDs that lack any recorded incidence or prevalence values in the initial RD catalog. Additionally, it is crucial to prioritize epidemiological research on diseases that may be included in future iterations of the RD catalog. By conducting thorough epidemiological raw data research and aggregating the data, we can not only provide valuable data references but also facilitate dynamic updates of the RD catalog (*287*). As the amount of epidemiological data continues to increase, additional high-quality information can be obtained in the future. The epidemiological data identified using this approach could be included in further studies. This approach will enhance the academic quality of our understanding and management of RDs in China.

5. Conclusion

This study aimed to enhance the academic quality of epidemiological information and drug marketing assessment for RDs in China, based on the two RD catalogs.

By providing a systematic and comprehensive data reference, this study contributes significantly to the understanding of epidemiological information and facilitates accurate assessments of the RD drug market.

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

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Review

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Systematic review of phenotypes and genotypes of patients with gastrointestinal defects and immunodeficiency syndrome-1 (GIDID1) (related to TTC7A)

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SUMMARY The objective was to conduct a comprehensive review of the morbidity and mortality observed in published patients with gastrointestinal defects and immunodeficiency syndrome-1 (GIDID1) related to TTC7A abnormalities. This included phenotypic, genotypic, and therapeutic aspects. Twenty-seven articles were included, which represented a total of 83 patients. Mortality was of 65.8% of the cases with a mean death at 11.8 months. The mortality rate was 197.1 per 1,000 patients-years, which is significantly higher than other enteropathy types caused by defects in epithelial trafficking and polarity (such as *MOY5B, STX3, EPCAM, SPINT2, TTC37* and *SKIV2L*). Prematurity was also significant, with an average gestational age of 34.8 weeks. Antenatal signs were observed in 30 patients, including 14 cases of hydramnios. Three distinct phenotypic associations were identified: immune deficiency and multiple intestinal atresia without enteropathy (ID/MI), immune deficiency and enteropathy without atresia (ID/E), and immune deficiency with multiple intestinal atresia and enteropathy (ID/MIA/E). The mortality rates for these groups were 91.6%, 47.3% and 55.5%, respectively ($p = 0.03$), at earlier age of mortality for the ID/MIA phenotype and a later one for the ID/E phenotype. ELA syndrome (Enteropathy, Lymphopenia and Alopecia) was only observed in the ID/E group. Among the three genotypes (double variant Nonsense NS/NS, variant Missense/Nonsense MS/NS, double variant Missense MS/MS), NS/NS was significantly associated with the ID/MIA phenotype (77.8%), while MS/MS was associated with the ID/E phenotype (73.7%). Few therapies have been shown to be effective in treating enteropathy, particularly immunosuppressive therapies and hematopoietic stem cell transplants. The use of Leflunomide in one patient did not yield successful treatment outcomes. In conclusion, we confirm association between mortality and phenotype, which is itself linked to genotype.

Keywords gastrointestinal defect and immunodeficiency-1, TTC7A, immune deficiency, enteropathy, intestinal atresia

1. Introduction

Gastrointestinal defects and immunodeficiency syndrome-1 (GIDID1) is a clinical condition that has been described since 1974 (*1*). It was initially characterized by the presence of multiple intestinal atresias in the context of consanguinity. However, it wasn't until 2013 that GIDID1 was specifically associated with an abnormality oh the TTC7A gene located on chromosome 2p21 (OMIM: 243150). This condition is inherited in an autosomal-recessive manner and can lead to both intestinal abnormalities such as apoptotic enterocolitis, multiple intestinal atresias, recurrent intestinal strictures (*2*), as well as immune deficiencies (*3*). Pathophysiologically, the loss function in the TPR domain results in abnormalities in protein interactions and transcription (*3-5*). In enterocytes, this leads to disruptions in apicobasal polarization due to the absence of regulatory control over the RhoA signaling pathway (*6*). In T cells, it impairs their proliferation,

adhesion, and migration capabilities within the thymus (*7*). These factors contribute to a state of continuous apoptotic enteropathy and lymphocyte depletion, resulting in reverse bipolarity (*8*).

The prognosis for individuals with this condition is extremely severe, with an elevated early mortality rate. In fact, more than half of children succumb to the disease before reaching their first year of life (*9*). GIDID1 remains a rare disorder, usually only documented in case reports or small series.

The objective of this study is to conduct a systematic review of published cases to provide insights into the various phenotypes and their evolution, the effectiveness of different therapeutic approaches, the correlation between phenotype and genotype, and the association with morbidity and mortality.

2. Systematic Review

We conducted a systematic review of published case involving patients with GIDID1. The search was conducted following the PRISMA methodology on NCBI, using the keywords "tetratricopeptide repeat domain 7A" and "TTC7A". The most recent search was conducted in July 2023. Articles containing clinical data were included, while those lacking phenotypic or genotypic information were excluded. For each patient, information on sex, antenatal manifestations, and anthropometric data at birth, clinical course, and therapies administered and their effectiveness, mutations, type of pathogenic variant, as well as morbidity and mortality, were collected.

The type of immune deficiency has been defined as follows (*10*):

i) CVID (Common Variable Immune Deficiency): Characterized by age-related hypogammaglobulinemia, with may be associated with normal or decreased circulating B cell counts, co-activation defects, or defects in B cell survival.

ii) CID (Combined Immune Deficiency): Defined by CD3 T-cell lymphopenia of less than 1000/µL before 2 years of age, less than 800/µL between 2 and 4 years of age, or less than 600/µL after 4 years. Il also includes lymphocyte proliferation capacity less than 30% of normal.

iii) SCID-Like (Severe Combined Immunodeficiency): Characterized by CD3 T-cell lymphopenia of less than 300/µL and lymphocyte proliferation capacity less than 10% of normal as measured by the PHA test. "SCID-Like" is preferred to the term "SCID" to distinguish it from primary SCID, which is not associated with gastrointestinal enteropathy.

Enteropathy was defined as diarrhea with more than 7 daily episodes persisting for more than 2 weeks, often presenting with bloody diarrhea (*11*).

Genotypes were categorized as homozygous or composite heterozygous, and based on their consequences on the protein, they were classified as Nonsense (NS) (indicating loss of function due to gain arrest, insertion, deletion, frame shift, splicing site, or long deletion) or Missense (MS).

The statistical analyses were carried out using the BiostatTGV software.

3. Main Findings

A total of 58 articles were identified, and 27 were included, encompassing a total of 83 patients (Prisma Flow Chart SI). Articles lacking phenotypic or genotypic information were excluded. Among these patients, 74 had pathogenic variations missense or nonsense in *TTC7A*, while the remaining 8 were siblings of affected individuals, exhibiting a concordant phenotypic presentation (*6,12*), and one had an in-frame long deletion mutation in exon 1 (c.133_154del) resulting in p.(Gly45_Ala55del), which could not be classified as either missense or nonsense variant. The sex ratio was approximately equal, and inbreeding was observed in 40.9% of cases, significantly more often associated with the ID/E phenotype ($p = 0.001$) (Table 1). The average follow-up period was 40.5 months, with the longest follow-up extending to 50 years, particularly for the ID/E phenotype.

3.1. Prematurity and antenatal signs

Prematurity was observed in 85.7% of cases, with an average gestational age of 34.8 weeks, often accompanied by eutrophic parameters. No significant differences were observed in terms of birth gestational age among different phenotypes (Figure 1). Antenatal signs were described in 30 patients, including 19 cases of intestinal dilatation/atresia, 14 cases of hydramnios/polyhydramnios, and 10 cases of intraluminal calcification. There was no significant difference between prematurity and the presence of hydramnios when compared to other antenatal signs. Similarly, there was no significant difference in prematurity between those who exhibited antenatal signs and those who did not.

3.2. Clinical description

Clinically, this condition manifested itself as early-onset diarrhea, with an average onset at an age of 8.6 months, with 32 cases considered. Digestive atresia could be located anywhere from the stomach to the anus, as specified in 45 cases, with a predominant occurrence in the small intestine (62.2%), followed by the colon (37.8%), leading to microcolon formation in 15.5% of cases, and in the pyloric stomach (35.5%) (Figure 2). These atresias were observed as both single in 4 cases (pyloric, ileum, anus) and as multiple occurrences in 41 cases (91.1%). Subsequent anatopathological studies

Table 1. Epidemiological and clinical description of patients with GIDID1 whole cohort and according to the 3 most common phenotypic combinations

Items	TOTAL *n* = 83	Combinations of phenotypes*			*p*
		ID/MIA *n* = 12	ID/E *n* = 19	ID/MIA/E *n* = 21	
Epidemiology					
Male / Female	40/35	7/4	10/9	6/12	0.24
Consanguinity: Y/N (%)	27/39 (40.9)	2/10 (16.7%)	14/4 (77.7%)	3/15 (16.7%)	0.0001
Age at last eval. in month	40.5	17.6	91.3	42.5	0.036
(range, *n*)	(0.03-600, *n* = 78)	(0.2-96, *n* = 12)	(5- 600, *n* = 19)	(1.2-228, *n* = 20)	
Dead/Alive	52/27 (65.8%)	11/1 (91.6%)	9/10 (47.3%)	11/9 (55.5%)	0.036
Death rate per 1000 patients-year	197.1	625.6	62.2	155.5	NA
Age at death in month	11.8	10.5	34.7	12.6	0.18
(range, *n*)	(0.06-168, *n* = 51)	(0.2-46, *n* = 11)	(5.6-168, *n* = 9)	(1.2-41, *n* = 11)	
Birth auxological data					
Birth weight percentil	31.7	22.5	39.3	35	0.89
(range, *n*)	(1.06-88, *n* = 12)	(1.06-44, *n* = 2)	(39-40, *n* = 3)	(20-50, *n* = 2)	
IUGR: weight < 10th percentil (*n*)	4 (*n* = 12)	1 (*n* = 2)	0	0	NA
Birth size percentil	21.8	38	20	26.3	NA
(range, *n*)	(0.7-50, *n* = 9)	(*n* = 1)	(*n* = 1)	(2.5-50, *n* = 2)	
IUGR: size < 10th percentile (*n*)	3 (*n* = 9)	0	0	1 (*n* = 2)	NA
Mean birth term	34.8	34.6	35.6	35	0.46
(range in weeks, *n*)	(23-40, *n* = 28)	(32-38, *n* = 3)	(35-36, *n* = 3)	(33-39, *n* = 9)	
Prematurity: Y/N	24/4 (85.7%)	2/1 (66.7%)	3/0 (100%)	8/1 (88.8%)	0.46
(mean, range in weeks)	(34, 23-36)	(33, 32-34)	(35, 35-36)	(34, 33-36)	
ID					
Total: Y/N	62/3 (95.4%)	12 (100%)	19 (100%)	21 (100%)	NA
Type ID					
CVID (Y%)	22 (35.5%)	2 (16.7%)	11 (57.9%)	7 (33.3%)	
CID (Y%)	14 (22.5%)	3 (25%)	8 (42.1%)	0	< 0.0001
SCID-Like (Y%)	26 (42%)	7 (58.3%)	0	14 (66.7%)	
Parenteral Nutrition					
Y/N (Y%)	49/4 (92.4%)	9/0 (100%)	9/4 (69.2%)	18/0 (100%)	0.009
Age at PN start	2,6	2,2	0,9	3,5	0.43
(range, *n*)	(0.03-7, *n* = 19)	(0.03-7, *n* = 5)	(0.3-1.4, *n* = 3)	(0.03-6, *n* = 10)	
Weaning: Y/N (Y%)	2/49 (3.9%)	0/8 (0%)	0/9 (0%)	1/13 (7.1%)	0.51
Age at weaning month	24	-	-	24	NA
(range, *n*)	(24, *n* = 1)			(*n* = 1)	
Treatments					
Surgery: Y/N (Y%)	34/2 (94.4%)	8/0 (100%)	0/2 (0%)	18/0 (100%)	0.003
HSCT: Y/N (Y%)	13/63 (17.1%)	2/10 (16.7%)	2/17 (10.5%)	8/13 (38.1%)	0.10
Efficiency HSCT: Y/N (Y%)	6/7 (46.1%)	0/2	0/2	5/3 (62.5%)	0.36

*Phenotype combinations are described in only 52 patients despite a total cohort of 83 patients.

confirmed the presence of apoptotic enterocolitis. Immune deficiency was present in 95.4% of cases and, on average, was diagnosed at an age of 18.4 months (as specified in a 54 cases).

The clinical course was characterized by recurrent atresias, which were described in 13 cases (*3-6,12-15*), recurrent infections in 37 cases, ELA syndrome (enteropathy, lymphopenia and alopecia) emerging from 2 years of age in 5 cases (*7,16,17*), and autoimmune diseases appearing in 12 cases at an average age of 3 years. The auto-immune conditions included auto immune thyroiditis (*n* = 1), auto-immune hemolytic anemia (*n* = 1), psoriasis (*n* = 1), type 1 diabetes (*n* = 2), auto-immune dermatitis (*n* = 1), auto-immune hepatitis (*n* = 1) and auto-immune gastritis (*n* = 1) or enteric (*n* = 3) (*7, 18-22*).

3.3. The three distinct phenotypes

Based on data from 55 patients and considering the three primary signs (multiple intestinal atresia, immune deficiency, enteropathy), three distinct phenotypic combinations were identified (Figure 3): immune deficiency and multiple intestinal atresia without enteropathy (ID/MIA), immune deficiency and enteropathy without atresia (ID/E), immune deficiency, multiple intestinal atresia and enteropathy (ID/MIA/E), with proportions 21.8%, 34.5% and 38.1%, respectively (more details provided in Table 1). Regarding genotypes, 63% were homozygous, with a notable prevalence of double missense (MS) variant mutations with phenotype ID/E ($p < 0.001$) (Table 2).

3.4. Clinical management

Parenteral nutrition (PN) was initiated in 92.4% of the cases (specified in a 51 cases), at an average age of 2.6

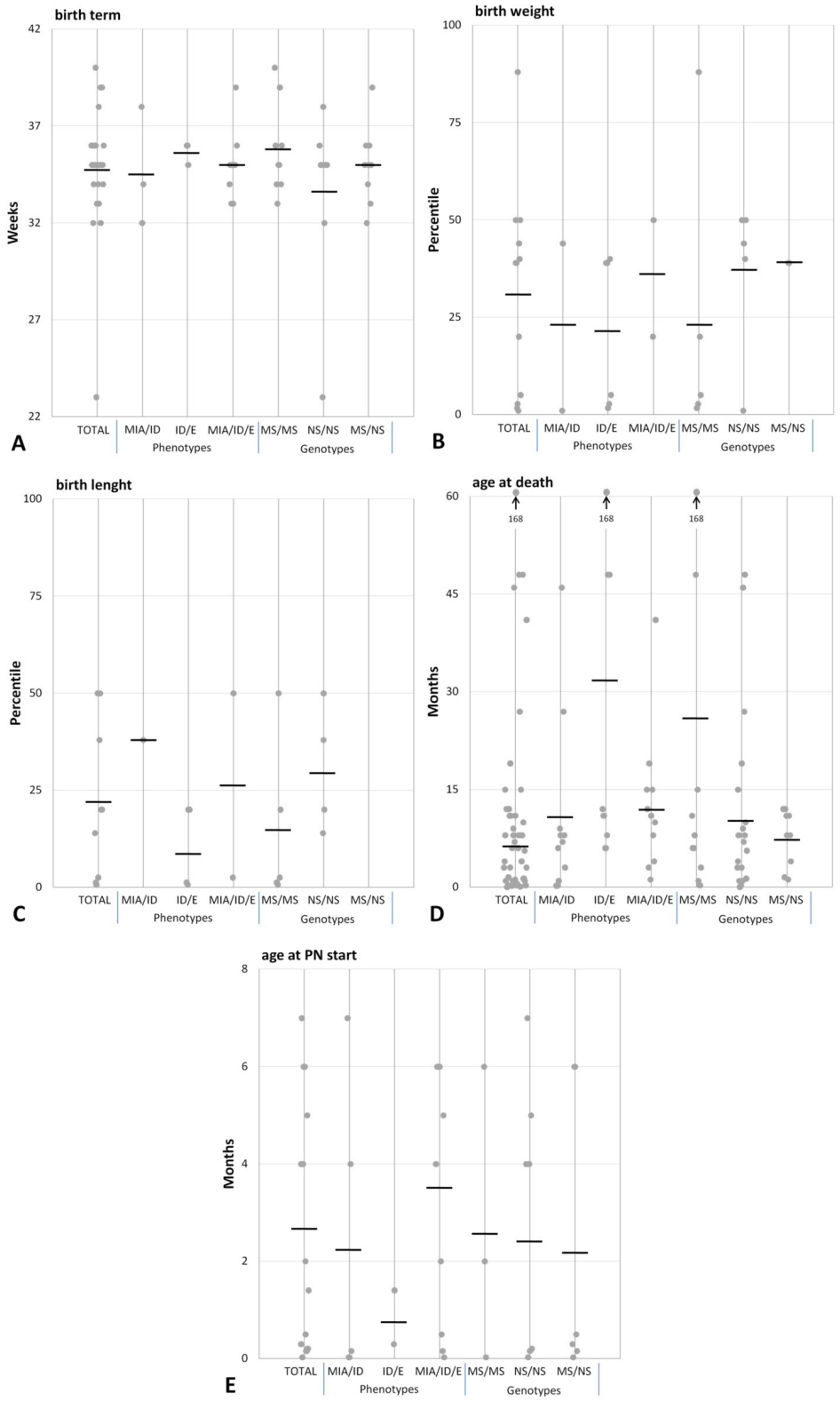

Figure 1. Median for term (A), birth weight (B) and height (C), age at death (D), and age at onset and end of parenteral nutrition (PN) (E) as a function of genotypes and phenotypes of patients with GIDID1.

months, and was successfully discontinued in only 2 cases, at 24 months in one case. The initiation of PN was early in cases of digestive atresia and delayed in cases without digestive atresia.

Immunosuppressants, such as corticosteroids, azathioprine or anti-TNF-alpha drugs, were used in 15 cases (*4,5,11,12,23,24*). They were found to be effective in alleviating diarrhea in only 2 cases using corticosteroids, azathioprine and anti TNF-alpha (*5,24*).

One case of severe pruritus at 3 years of age proved unresponsive to multiple therapies including corticosteroids, histamine blockers, gabapentin,

clonidine, mirtazapine, amitriptyline, and mepolizumab (anti-IL5). It could be successfully treated with Dupilumab (anti-IL4) at the dosage of 100 mg subcutaneously every 2 weeks (*22*).

Hematopoietic Stem Cell Transplantation (HSCT) was performed in 13 cases, with a median age of 8.6 months, resulting in an extension of survival in 5 cases (38.5%) (*3,4,6,7,12,14,17,18*). No intestinal/liver transplants were performed successfully ($n = 1$) (*17*).

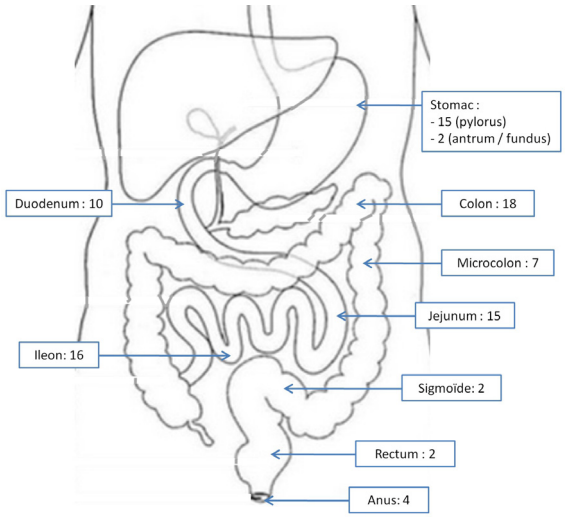

Figure 2. Affected digestive segments in MIA of patients with GIDID1 ($n = 45$), of whom 41 have multiple atresias, and 4 have single (1 pylorus, 1 ileum, 2 anus).

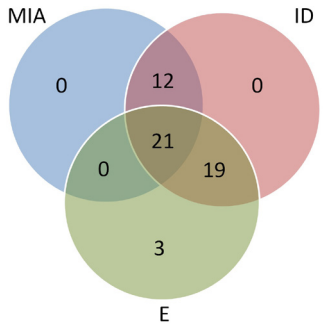

Figure 3. Venn diagram of the 3 combinations most common phenotypic results of patients with GIDID1 ($n = 55$).

Treatment with Leflunomide, initiated at 4 months of age, was found to be ineffective (*25*).

3.5. Mortality

Mortality was recorded at 65.8%, with a mean age at the time of death being 11.8 months. It was notably severe and occurred early, with 82.3% of the cases resulting in death before the age of 12 months ($p < 0.05$). The mortality rate was 197.1 per 1,000 patients-years (Table 3). The survival curve based on the most frequent phenotypic associations revealed that the rates for these groups were 91.6%, 47.3%, and 55.5%, respectively ($p = 0.03$), with an earlier mortality age for the ID/MIA phenotype and a later one for the ID/E phenotype (Figure 1, Figure 4, and Table 1). The causes of mortality were provided for 35 cases. The primary cause was infectious

Table 3. Death rate per 1000 patients-year of enteropathy caused by defect of epithelial trafficking and polarity (MYO5B, STX3, EPCAM, SPINT2, TTC37, SKIV2L and TTC7A) or by enteroendocrine cell dysfunction (PCSK1 and NEUROG3), and smooth muscle disorders (ACTG2, MYH11, FLNA, RAD21)

Gene	Death reppored	Total patients described	Death/year of follow up for 1,000
STX3	0	3	0
FLNA	0	7	0
MYH11	2	16	3.52
RAD21	1	3	11.6
ACTG2	20	65	13.5
EPCAM	11	71	15
NEUROG3	2	14	13.9
SKIV2L	2	12	24.1
PCSK1	5	32	22.8
TTC37	8	36	52.3
MYO5B	12	43	53.1
SPINT2	14	35	76.5
TTC7A	52	79	197.1
TTC7A ID/MIA	11	12	625.6
TTC7A ID/E	9	19	62.2
TTC7A ID/MIA/E	11	20	155.5
TTC7A MS/NS	9	17	340.7
TTC7A NS/NS	23	25	784.1
TTC7A MS/MS	12	28	61.1

Data from this study, 32 and 33. Detail according phenotype and variant combination for TTC7A.

Table 2. Genetic phenotypic description of patients with GIDID1 whole cohort and according to the 3 most common phenotypic combinations

Items	Total $n = 84$	Combinations of phenotypes*			p
		ID/MIA $n = 9$	ID/E $n = 19$	ID/MIA/E $n = 21$	
Homozygous	53 (63%)	4 (44.4%)	12 (63.1%)	4* (20%)	0.020
Composite heterozygous	31 (37%)	5 (55.5%)	7 (36.9%)	16* (80%)	
MS/NS	17 (20.2%)	1 (11.1%)	3 (15.8%)	9* (45%)	0.0007
NS/NS	28 (33.3%)	7 (77.8%)	2 (10.5%)	5* (25%)	
MS/MS	39 (46.4%)	1 (11.1%)	14 (73.7%)	6* (30%)	

*Mutations are described in only 50 patients despite a total cohort of 83 patients. Moreover, it was not possible to define variant type for 2 cases (*18*).

diseases (n = 14, 40%), followed by post-therapeutic complications (n = 8, 22.9%), occlusive issues (n = 7, 20%), and liver pathologies (liver failure and portal hypertension) in 4 cases, with 3 of them requiring parenteral nutrition. Finally, 2 cases (5.7%) were attributed to enteropathy. Deaths due to post-therapeutic complications occurred after hematopoietic stem cell transplantation (HSCT) in 8 out of 13 HSCT procedures performed. The survival curve based on genotypes (Figures 5 and 6) revealed a poor prognosis for patients with double NS variant, with over 77% of them passing away before the age of 10 months, and 92% of patients succumbing by the end of the follow-up period, at the latest, by 96 months.

3.6. Subgroup comparison

When the groups were compared, significant differences were observed. A notable difference in mortality was observed, with early severity in combinations involving gastrointestinal atresia (p < 0,05), particularly the ID/MIA combination, which exhibited severe mortality (91.6%) and an average age of death of 10.5 months (Table 1). This combination was more frequently associated with double NS variant (p < 0.001). The follow-up duration was longer for ID/E, with an average of 91.3 months (p < 0.05), and this phenotype showed a later age of death (p < 0.05). Inbreeding was significantly associated with the ID/E phenotype (p = 0.001), as well as ELA syndrome, which was exclusively present in this phenotypic combination, occurring in 3 families and encompassing a total of 5 cases (*7,16,17*). In 4 cases, these individuals also presented with onychopathies, such as distal sub-ungual hyperkeratosis of the nails (*7,16,17*). In ELA syndrome, enteropathy typically emerged within a few days of life, followed by lymphopenia between

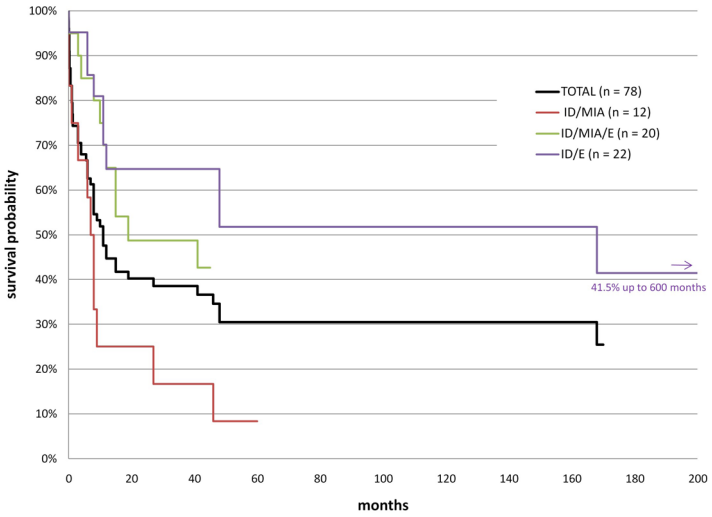

Figure 4. Survival curve of patients with GIDID1 as a function of all cases (n = 78) and the 3 most frequent phenotypic combinations (n = 54).

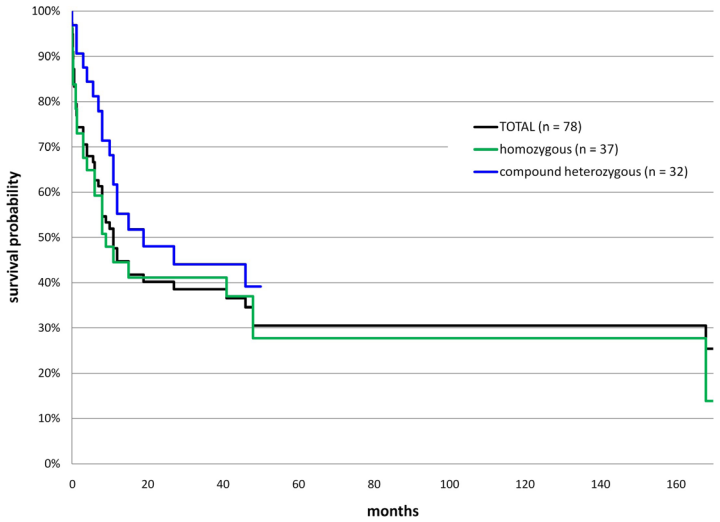

Figure 5. Survival curve of patients with GIDID1 as a function of all cases (n = 78) and homozygous or composite heterozygous genotype (n = 69).

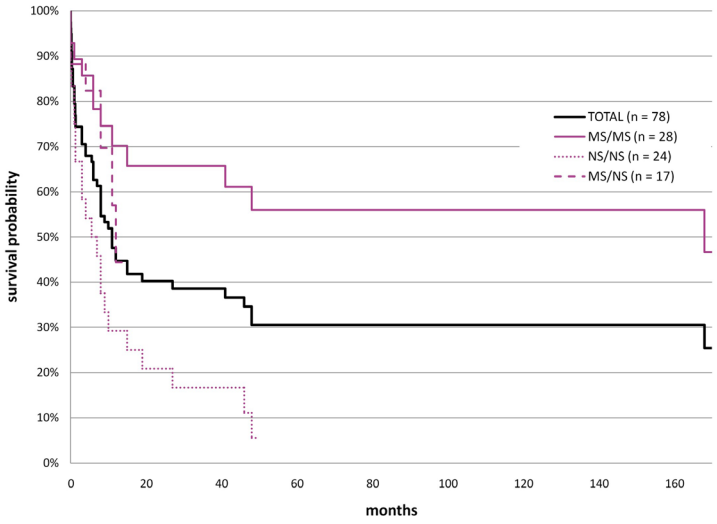

Figure 6. Survival curve of patients with GIDID1 as a function of all cases (*n* = 78) and combination of variants MS: missense, NS: nonsense (MS/MS, MS/NS, NS/NS) (*n* = 69).

6 months and 3 years, and alopecia starting at 2 years of age, leading to progressive hair loss and complete alopecia, including eyebrow loss.

Surgery was often performed in cases with the atresia phenotype, as well as parenteral nutrition ($p < 0.01$), which notably started earlier. The use of HSCT is more frequently associated with ID/MIA/E, without a significantly better efficacy (Table 1).

Regarding genotype/phenotype correlation, when comparing our three phenotypic associations to genotypic combinations involving double MS variant, double NS variant, and MS/NS variant, a significant difference was observed (Table 2). The ID/MIA association was strongly associated with double NS variant (77.8%), while the ID/E association was more strongly associated with double MS variant (73.7%) ($p < 0.001$). The ID/MIA/E group exhibited intermediate characteristics. Detailed phenotypic and genotypic characteristics are provided in Tables 1 and 2.

4. Discussion

Our literature review included a total of 83 cases, one-third more than the previous systematic review published in 2019 by Jardine *et al.* (*2*), which included only 55 cases.

In the cohort of patients we observed, we confirmed several previously highlighted elements: the presence of three primary phenotypic traits - MIA, ID and E (*23*); a high mortality rate, with 82.3% of deaths occurring before the age of 12 months, reaffirming the severity of this disease (*3-7,12,14,15,23,25,26*), and the crucial role of parenteral nutrition (PN), as 92.1% of patients required it (*3,5-7,11-15,17,18,22-29*).

4.1. GIDID1 is associated with preterm birth

Furthermore, we identified phenotypic associations and a connection with prematurity, with an average gestational age of 34.8 weeks. Notably, this prematurity did not appear to be related to the presence of antenatal signs and therefore seems to be intrinsic to TTC7A deficiency. Although the gestational term was not provided for 55 patients in our cohort, even if all of them had been born at term, it would still represent 28.9% of preterm births, exceeding the global population's prematurity rate, which stood at 11.1% in 2010 (*30*).

We have showed that the presence of inbreeding is more significant for the DI/E association without MIA but this result is influenced by the existence of a family of 14 patients with this phenotype (*7*).

4.2. Survival difference according genotype

Differences in survival based on genotype have been previously suggested (*9,23*), and our study confirms this hypothesis. Specifically, we found that patients with a double MS variant exhibited better survival, whereas double NS variant patients had more frequent and earlier mortality, with the latter being more commonly associated with digestive atresia. This supports the idea that a double MS variant, likely a hypomorphic form, allows for better survival due to residual protein levels supporting essential cell functions. Also, when compared to certain enteropathies caused by defect in epithelial trafficking and polarity (*EPCAM, MYO5B, STX3, TTC37, SPINT2* and *SKIV2L*) enteropathy caused by enteroendocrine cell dysfunction (*PCSK1* and *NEUROG3*) or CIPO (Table 3), the mortality rate per 1,000 patient-year was notably higher, especially for the ID/MIA phenotype and the double NS variant (*31,32,33*).

4.3. Strong genotype-phenotype association

By comparing our three phenotypic associations to the genotypic associations of double MS, double NS and

MS/NS variant, we observed a significant difference: the ID/MIA category being strongly associated with double NS variant, whereas the ID/E category showed a stronger association with double MS variant It is challenging to determine whether the observed differences in survival are linked to genotype or phenotype, as these two factors are intrinsically interconnected. It appears that double NS variant is more commonly associated with intestinal atresia. This suggests that the complete absence of TTC7A has a more profound impact on the structure of the digestive tract (*3-5,11,14,15,28,34*), while a hypomorphic presence allows for a normally developed gastrointestinal tract but leads to a phenotype resembling very early onset-inflammatory bowel disease (VEO-IBD) (*3-5,7,10,14,25*). Additionally, the ELA syndrome (*7,16*) was exclusively linked to patients without intestinal atresia. This could imply either a correlation with specific genotypic variant, such as double MS variant, or the fact that it requires longer survival to manifest (average onset at 24 months).

4.4. No clearly effective therapeutic

Regarding therapeutic approaches, we suggest the ineffectiveness of HCST on enteropathy (*2,18,35*) although it allows for prolonged survival in over half of the cases for the ID/MIA/E phenotype. Surgeries were generally performed in cases of atresia, and we highlighted recurrent atresia (n = 13), which, in some instances, warranted multiple surgeries and could result in a short bowel syndrome (*3-6,12-15*). Leflunomide treatment, suggested to improve TTC7A deficiency in *in vitro* models (*2*) by reducing cellular apoptosis, did not yield positive results in the one patient treated, who happened to have a double NS variant (*25*). However, there is some indication of a positive, as one patient reported on the Boston Children's Hospital website showed improvements in enteropathy. Further observational studies are required to better evaluate the efficacy of leflunomide for specific mutation subtypes.

4.5. Limitations

We conducted an extensive literature review, thereby increasing our sample size by one-third compared to the previous cohort, thus enhancing our understanding of this complex pathology. However, limitations stem from a lack of data and details in some case, as the results were extracted from published sources. When information on the type of immune deficiency or the presence of the enteropathy was lacking, we made clarifications based on objective criteria published in 2014 (*10*). Cases with missing information on immune deficiency were categorized as "absence of data", as were cases lacking data of enteropathy.

It should be noted that a potential bias exists due to the presence of a published family comprising 14 patients (16,9% of cohort) (*7*), all of whom had double MS variant, likely hypomorphic. This bias impacts the ID/E phenotype or the double MS variant subgroup.

As with all rare diseases, it is crucial to publish cases with rigorous criteria to establish a more precise understanding of the disease's progression and effectiveness of therapeutic approaches.

5. Conclusion

Our literature review reaffirms the severity of the GIDID1 associated with TTC7A mutations. It verifies the existence of three major phenotypic associations and enriches our knowledge, particularly in terms of genotype correlation: a more severe prognosis is associated with the presence of digestive atresia and double NS variant. We also highlight the link between GIDID1 and prematurity, which had not been previously described. Further studies with detailed characterization of both phenotype and genotype are essential to enhance our understanding of this condition, including its phenotypic and therapeutic aspects.

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Mini-Review

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Single-cell metabolomics in rare disease: From technology to disease

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SUMMARY With the development of clinical experience and technology, rare diseases (RDs) are gradually coming into the limelight. As they often lead to poor prognosis, it is urgent to promote the accuracy and rapidity of diagnosis and promote the development of therapeutic drugs. In recent years, with the rapid improvement of single-cell sequencing technology, the advantages of multi-omics combined application in diseases have been continuously explored. Single-cell metabolomics represents a powerful tool for advancing our understanding of rare diseases, particularly metabolic RDs, and transforming clinical practice. By unraveling the intricacies of cellular metabolism at a single-cell resolution, this innovative approach holds the potential to revolutionize diagnosis, treatment, and management strategies, ultimately improving outcomes for RDs patients. Continued research and technological advancements in single-cell metabolomics are essential for realizing its full potential in the field of RDs diagnosis and therapeutics. It is expected that single-cell metabolomics can be better applied to RDs research in the future, for the benefit of patients and society.

Keywords rare diseases, single cell omics, inborn errors of metabolism, single-cell metabolomics

1. Introduction

Rare diseases (RDs) are characterized by their low prevalence in the general population (*1*). Currently, around 6,000-8,000 RDs have been identified, but there are still many undiagnosed and unknown diseases (*2*). This poses a growing public health concern, particularly because the majority (50-75%) of RDs primarily affect children, and a significant proportion (approximately 80%) have a genetic basis (*3-5*).

The prevalence of RDs can vary significantly depending on the region and specific disease type (*6*). The European Union Regulation on orphan medicinal products defines RDs as diseases that affect fewer than 1 in 2,000 individuals in Europe. Similarly, the American Orphan Drug Act defines RDs as diseases that affect fewer than 200,000 patients in the United States. In China, it is estimated that there are approximately 20 million RD patients (*6*). It is important to note that the exact morbidity for most RDs is not currently available, highlighting the need for further research and awareness in this field. Metabolic related diseases comprise a significant portion of rare diseases, representing the majority (*7*). These diseases encompass inborn metabolic abnormalities as well as other rare metabolic conditions with low incidence in the general population (*8*). Inborn errors of metabolism (IEMs) are a subset of rare metabolic diseases that result from defects in enzymes, co-factors, or transport proteins due to mutations affecting crucial metabolic enzymes (*8*). Currently, there are more than 1450 known types of IEMs (*9*). It shows low prevalence but high death rate in IEMs patients. A study estimated the global birth prevalence of all-cause IEM, which is 50.9 per 100,000 live births, resulting in almost 0.4% of child deaths worldwide in 2018 (*10*).

Over the years, there have been significant advancements in basic research, clinical case registration, and the development of orphan drugs for rare diseases (*11,12*). However, it is widely recognized that patients with rare diseases face significant challenges in accessing a definitive diagnosis and effective treatment, particularly in many regions of the world (*13*). The widespread use of next-generation sequencing technology has had a transformative impact on diagnostic accuracy

and cost-effectiveness, surpassing older technologies (*14,15*). Exome sequencing (ES) has played a crucial role in identifying previously unknown diseases as rare diseases (*16,17*). As integrated technologies, such as genomic, transcriptomic, metabolomic, proteomic, and methyl profiling analyses, are increasingly considered for clinical use, functional studies should be conducted to facilitate efficient diagnosis and treatment of rare diseases (*18-21*).

Metabolomics is a field of study that focuses on the analysis of metabolites, including amino acids, sugars, and lipids (*22*). These metabolites have been shown to play vital roles in cellular signaling and various biological processes (*23*). Different from proteomics and genomics, metabolomics provides insights into real-time biochemical activity (*22*). By analyzing metabolomics datasets, researchers can uncover relationships between cellular activities, metabolic processes, and biological mechanisms in both health and disease (*24*). There are three commonly used categories of analytical workflows in cell metabolism analysis: testing the general inputs and outputs of metabolism, characterizing metabolic enzymes through enzyme activity assays, and utilizing steady-state metabolomics analysis through mass spectrometry (MS) technology (*25*). Single-cell technology enables qualitative and detailed analysis of the extensive molecular information carried by a large number of biomolecules at the single-cell level (*26*). Single-cell metabolomics can identify phenotypic heterogeneity between individual cells and discover seemingly similar cell subpopulations to decipher disease specificity, explore stage differences in disease progression, and provide evidence for disease treatment.

Capillary electrophoresis electrospray ionization (CE-ESI) is one of the new techniques for single-cell metabolite analysis (*27*). This technique allows for in situ micro-sampling of live single cells, eliminating the need for cell dissection and separation. CE-ESI bridges the technical gap between comprehensive non-targeted metabolomics and live single-cell analysis (*28*). Another technique, probe-based electrospray ionization (ESI), has been specifically designed for in situ single-cell metabolite analysis (*28*). The development of above techniques offers dual benefits: reduced detection limitations resulting by low sample dilution and the ability to recognize unknown molecules. In the field of single-cell proteomics by mass spectrometry (SCoPE-MS), single cells are amplified to generate sufficient signals for peptide sequencing using tandem mass tags (TMT) (*29*). Looking ahead, we can expect more advanced labeling workflows to be developed for exploring single-cell metabolomics.

2. Single-cell metabolomics in IEM

IEM can affect various organs throughout the body, and the clinical manifestations vary among patients,

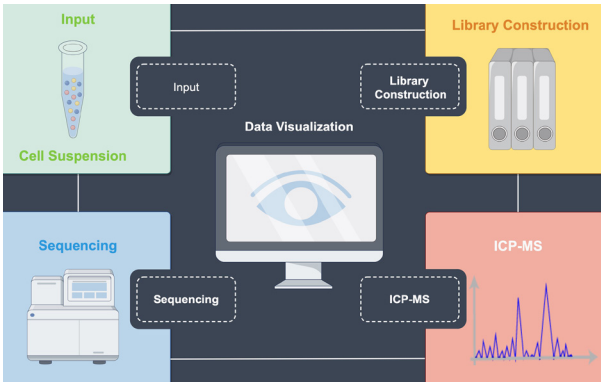

Figure 1. The work flow of single cell sequencing technology combined with Inductively Coupled Plasma Mass Spectrometry (ICP-MS) technology.

often lacking specificity (*10*). This makes it challenging to determine when to perform IEM testing and which specific laboratory tests to conduct due to the absence of characteristic signs and symptoms. However, the development of tandem mass spectrometry (MS) has significantly improved the detection capability for a wider range of diseases from a single blood spot (*30*). Previously, MS was used to test blood and urine samples, relying on the detection of metabolites that are indicative of specific diseases. If the results of metabolite testing suggest a potential disorder, Sanger or next-generation sequencing can be employed to confirm the diagnosis (*31*).

Metabolomics testing is crucial because it is often quicker than other methods, providing valuable information about disease severity and helping to elucidate the significance of mutations found in transcriptome sequencing (*32*). In recent years, numerous methods of analysis have been improved to enable single-cell level analysis, basing on inductively coupled plasma mass spectrometry (ICP-MS) (*33*). The short dwell time of cells in single-cell ICP-MS, which lasts only milliseconds, allows for precise measurement of single-cell metals (*34,35*) (Figure 1). Single-cell ICP-MS has become increasingly important in studying the metal-related properties of cells and its role in investigating cell metal-drug penetration in drug research (*36*).

In addition to single-cell ICP-MS, another valuable concept in sample introduction is the plotting of single cells on a flat surface, which facilitates qualitative and quantitative analysis at the single-cell and sub-cellular levels (*37*). With advancements in high sensitivity and spatial-temporal resolution imaging, researchers now have the ability to conduct detailed qualitative and quantitative analyses at these specific levels (*37,38*). Combination of high-resolution imaging of Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS) and single-cell technology prompted new generations of agents based on metals in precision medicine (*39,40*).

Leigh syndrome (LS) is a rare and devastating

early mitochondrial disease which primarily infringes infants and young children. It is considered one of the most severe mitochondrial diseases in children, which belong to the largest class of IEM (*41*). The prevalence of LS is estimated to be around 1 in 36,000 newborns (*42*). Unfortunately, the limited number of patients and the lack of validated disease models have hindered the exploration of potential mechanisms of LS neuronal pathology (*43*). The scarcity of treatments and medications for LS results in a high mortality rate, with many patients not surviving past three years of age (*44*). In an effort to shed light on LS pathology and explore potential therapeutic strategies, a study utilized patient-derived induced pluripotent stem cells and CRISPR/Cas9 engineering to develop workable human LS model. By integrating single-cell multi-omics analysis, the study uncovered abnormal metabolic states in neurons derived from mutant nerve cultures and brain organoids. It was discovered that metabolic defects caused by mutations in the SURF1 gene, a key gene in LS, disrupt the ability of differentiated cells to maintain proliferative and glycolytic states, leading to impaired neuronal morphogenesis and maturation. Single-cell metabolomics data played a crucial role in revealing the importance of metabolic programming in LS (*45*). As technology continues to advance and its application expands, single-cell metabolomics holds promise for exploring various diseases (Figure 2).

3. Single-cell metabolomics in other metabolism-related RDs

Inflammatory breast cancer (IBC) is a rare and aggressive form of breast cancer, which accounts for 1% to 5% of breast cancer cases and is associated with a higher death rate of 8% to 10% (*46*). A study investigating metabolic heterogeneity in IBC found elevated levels of N-acetylaspartic acid, a major metabolite (*47*). This analysis was conducted using clinical IBC samples and IBC cell line models, and it incorporated single-cell metabolomics techniques. The findings of this study revealed the crucial role of JAK2/STAT3 signaling in IBC resistance and identified potential biomarkers and therapeutic targets for IBC (*48*). These results demonstrate the potential of single-cell metabolomics in unraveling the metabolic characteristics and underlying mechanisms of IBC, providing valuable insights for the development of personalized treatment strategies and improving patient outcomes.

4. The future feasible application of new single-cell metabolomics technologies in RDs

In a recent study, a novel microfluidic device using surfacing enhanced Raman spectroscopy (SERS) was reported. This device enables the dynamic screening of single circulating tumor cells (CTCs), thereby providing valuable insights into the differential expression of multiple protein biomarkers in response to therapy (*51*). This automated detection technology holds immense clinical significance in the diagnosis and therapeutic efficacy monitoring of RDs. By enabling the detection and analysis of single CTCs, it offers a powerful tool for understanding disease heterogeneity from the single-cell perspective. This advancement has the potential to significantly improve our understanding of RDs and aid in the development of personalized treatment strategies. The integration of microfluidics and SERS in this device provides a practical and efficient method for screening and analyzing single cells, paving the way for future advancements in the field of single-cell analysis, especially in RDs researches.

5. Conclusion

Metabolomics plays a crucial role in systems biology and the study of various diseases. It combines emerging analytical tools with bioinformatics methods to study the role, abundance, content, downstream pathways and distribution of metabolic molecules in organisms. This

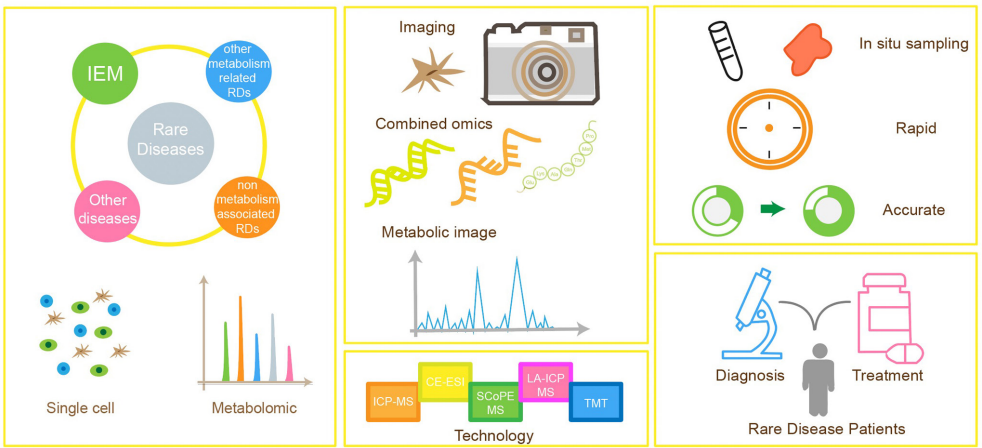

Figure 2: The use of single-cell metabolomics in rare diseases.

approach has proven to be valuable in understanding the mechanism of action and facilitating the diagnosis, including rare diseases (RDs). Single-cell metabolomics is an emerging field that focuses on analyzing the metabolome of individual cells to gain greater insights into cellular heterogeneity and disease processes in RDs. It has the potential to significantly enhance the ability to study the single-cell metabolome of RDs.

Integrating cellular omics, such as transcriptomics, peptidomics, and proteomics, is an important current goal in single-cell metabolomics research. By utilizing both single-cell transcriptome and metabolome measurements, researchers can assess the fit of disease models and identify models that closely resemble the patient's condition. This integration provides valuable insights into the details of gene transcription, translation, protein modifications, and metabolite interactions, enabling a better understanding of cell phenotype and fate. Overall, single-cell metabolomics is a rapidly evolving field which holds great promise for mastering RD-related knowledge, improving diagnostics and treatment strategies in the future.

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Original Article

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A novel mutation in the *OTOF* gene in a Chinese family with auditory neuropathy

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SUMMARY Gene therapy for monogenic auditory neuropathy (AN) has successfully improved hearing function in target gene-deficient mice. Accurate genetic diagnosis can not only clarify the etiology but also accurately locate the lesion site, providing a basis for gene therapy and guiding patient intervention and management strategies. In this study, we collected data from a family with a pair of sisters with prelingual deafness. According to their auditory tests, subject II-1 was diagnosed with profound sensorineural hearing loss (SNHL), II-2 was diagnosed with AN, I-1 was diagnosed with high-frequency SNHL, and I-2 had normal hearing. Using whole-exome sequencing (WES), one nonsense mutation, c.4030C>T (p.R1344X), and one missense mutation, c.5000C>A (p.A1667D), in the *OTOF* (NM_001287489.1) gene were identified in the two siblings. Their parents were heterozygous carriers of c.5000C>A (father) and c.4030C>T (mother). We hypothesized that c.5000C>A is a novel pathogenic mutation. Thus, subject II-1 should also be diagnosed with AN caused by *OTOF* mutations. These findings not only expand the *OTOF* gene mutation spectrum for AN but also indicate that WES is an effective approach for accurately diagnosing AN.

Keywords auditory neuropathy, *OTOF* gene, whole-exome sequencing

1. Introduction

Auditory neuropathy (AN) is a unique auditory disease in which patients can hear sounds but cannot comprehend them. AN involves both environmental and genetic factors, with more than 40% of cases caused by genetic factors (*1*). More than 20 genes have been reported to be related to AN (*2-4*). The lesion sites of AN include inner hair cells (IHCs), ribbon synapses, spiral ganglion neurons (SGNs) and demyelinated nerve axons. With the influence of time and the environment, the cells adjacent to the initial lesion site, including the outer hair cells (OHCs), will also deteriorate (*5,6*). Different pathogenic genes play a role in different parts of the auditory conduction pathway, and accurate molecular typing of AN can be performed through the analysis of pathogenic genes (*2,7-9*). The *OTOF* gene is the most common pathogenic gene for AN in different countries, and the mutation rate is as high as 41.2% in China (*10*). The main feature of AN caused by *OTOF* gene mutations is ribbon synapse lesions of IHCs, so this condition is also called auditory synaptopathy (*5,11*). Ribbon synapses are located between IHCs and primary afferent SGNs and participate in the transmission and coding of acoustic signals through exocytosis (*12*).

As a better understanding of the mechanism of AN is obtained, accurate diagnosis, intervention and effective treatment and rehabilitation have become topics of interest in the field of otology (*13-15*). The cost of developing gene therapy is extremely high, and its success depends on the accurate diagnosis of the disease by one or more specialized diagnostic tests. To date, gene therapy for monogenic AN has successfully improved hearing function in mice deficient in various target genes, such as *VGlut3*, *Otof*, and *Pjvk* (*13-15*). Appropriate candidates for gene therapy are determined by diagnostic tests, after which patients with specific defects can be treated. Thus, accurate genetic diagnosis can locate lesion sites and clarify the etiology to provide a basis for gene therapy and better guide patient intervention and management strategies (*1,16-18*).

In this study, we identified a family that potentially exhibited autosomal recessive hereditary hearing loss (HL). All the descendants in this family suffer from profound HL. To further explore the pathogenic genes of the affected individuals, we used whole-exome

sequencing (WES) to obtain mutation sites, which were subsequently screened and verified.

2. Materials and Methods

Written informed consent was obtained from the parents. The protocol was in accordance with the principles of the Declaration of Helsinki and was approved by the Ethics Committee of Beijing Tongren Hospital, Capital Medical University (Approval number: TREC2022-KY008).

2.1. Subjects

Data from a family of two sisters with prelingual deafness was collected from the Department of Otolaryngology, Head and Neck Surgery, Beijing Tongren Hospital (Beijing, China). The proband (II-2) was a 1 year old and passed the newborn hearing screening for otoacoustic emission (OAE) at birth. When she was six months old, her parents noticed that her hearing was poor, and her 12-year-old sister (II-1) exhibited similar symptoms. Patient II-1 had been wearing bilateral hearing aids for more than 10 years, but the effect was not satisfactory, as she could not speak clearly or hear easily. The father (I-1) of the participants was exposed to noise for a long time because of work, and the mother (I-2) reported normal hearing (Figure 1A).

2.2. Auditory tests and magnetic resonance imaging of the inner ear

The auditory tests included auditory brainstem response (ABR), distortion product otoacoustic emission (DPOAE), auditory steady-state response (ASSR), acoustic immittance, behavioral hearing and pure tone audiometry (PTA) tests. Acoustic immittance (226 Hz) was classified as A (including As and Ad), B, or C, where A was considered normal. The amount of 10% chloral hydrate solution used was determined according to the weight of subject II-2, and the ABR, ASSR and cochlear microphonic (CM) tests were performed while the patients slept. When the maximum sound output evoked no response, the default could not be determined. The hearing threshold was calculated as the average hearing level of PTA at 0.5, 1.0, 2.0, and 4.0 kHz according to the World Health Organization standard (2021) (*19*).

Magnetic resonance imaging (MRI) scans were obtained on a 1.5T GESigna scanner with matched eight-channel phased array coils. The protocol was designed to obtain routine axial and coronal unenhanced T2-weighted images and axial T1-weighted images, as well as axial three-dimensional fast imaging employing steady-state acquisition images of temporal bones. The cochlear nerve was also evaluated *via* MRI.

2.3. Whole-exome sequencing and variant analysis

Genomic DNA was extracted from the whole blood of four subjects and subjected to exome capture using an Agilent liquid capture system (Agilent SureSelect Human All Exon V6) according to the manufacturer's protocol. The DNA libraries were subsequently sequenced on an Illumina HiSeq sequencing platform to obtain paired-end 150 bp reads. The average sequencing depth of the target region was 127.00×, and the average coverage was 99.33%. Valid sequencing data were mapped to the reference genome (GRCh37/hg19) by BurrowsWheeler Aligner (BWA) software (*20*) to obtain the original mapping results in Binary Alignment/Map format. SAMtools (*21*) mpileup and BCFtools were used to perform variant calling and identify single-nucleotide polymorphisms (SNPs) and insertions/deletions (InDels). CoNIFER (*22*) was used to detect copy number variants (CNVs). ANNOVAR (*23*) was used to annotate SNPs, InDels and CNVs. The variant position, variant type, conservative prediction and other information

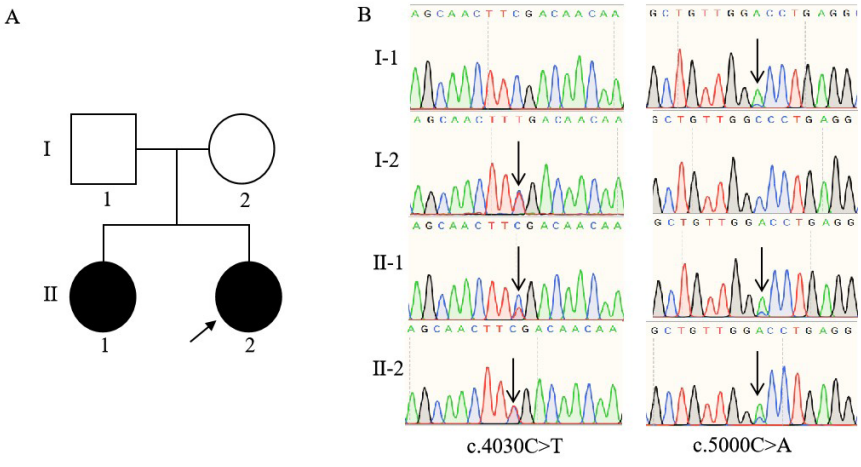

Figure 1. Pedigree and sequence analysis of *OTOF* mutations in the family. (A) Pedigree map of this family. **(B)** In this family, the compound heterozygous mutations c.4030C>T and c.5000C>A were observed in both affected siblings (II-1 and II-2); the c.5000C>A mutation was inherited from the father (I-1), and the c.4030C>T mutation was inherited from the mother (I-2).

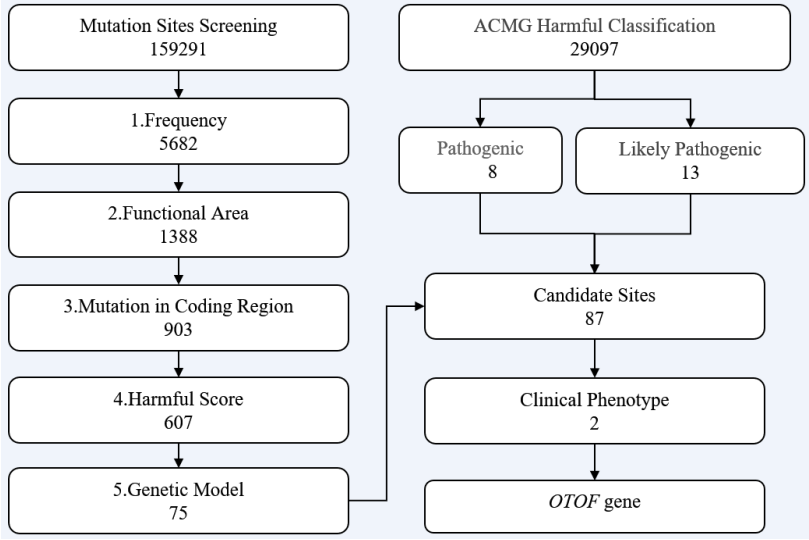

Figure 2. Variant screening process. 1. At least one mutation with a frequency higher than 1% was selected from four frequency databases: 1000g_all, ESP6500, gnomAD_ALL and gnomAD_EAS. 2. Variations in the coding region or splicing region (upper and lower 10 bp) were retained. 3. Synonymous SNP mutations not located in highly conserved regions that were not predicted by software to affect splicing and frameless InDel mutations of small fragments (< 10 bp) in the repeat region were removed. 4. Mutations were retained if they met one of the following conditions: a) predicted to be harmful or b) predicted to affect splicing. 5. Dominant inheritance: the sites with heterozygous mutations (mutation sites in sex chromosomes) in the patient's autosome and no mutations in healthy people in the family were selected as candidate sites; recessive inheritance: genes with at least two heterozygous mutation sites in patients were selected; the distribution of mutation sites on this gene in patients cannot be the same as that observed in any healthy person or a subset of the mutation sites in any healthy person. 6. Pathogenic and likely pathogenic mutations according to the ACMG guidelines were selected. 7. Steps 5 and 6 were combined to obtain candidate mutations. 8. The mutations that cosegregated with the phenotype in the pedigree were analyzed.

were obtained through a variety of databases, such as dbSNP (version 154), 1000 Genomes, GnomAD v2.1.1, CADD and HGMD. According to the American College of Medical Genetics and Genomics (ACMG) guidelines for sequence interpretation established in 2015 with the Human Genome Variation Society (HGVS) nomenclature, the mutations were categorized as pathogenic, likely pathogenic, uncertain significance, likely benign or benign. We used the existing database, software and ACMG guidelines combined with the subjects' clinical information to obtain candidate causative mutations (Figure 2). T-coffee (*24*) was used to analyze the conservation of the novel causative mutation in different species.

2.4. Sanger sequencing

The potential causative variants in this family were confirmed by Sanger sequencing of the amplified PCR products. We chose paired-end sequencing of the upstream and downstream regions of the mutation sites. The sequences of the primers used for the mutation sites in the *OTOF* gene are shown in Table 1. Each DNA sample was diluted to 20 ng/μL and used as a PCR template for amplification with Tsingke 1.1×T3 Super PCR Mix. The amplified PCR products were subjected to agarose gel electrophoresis (2 μL sample + 6 μL bromophenol blue), and the identification gel was visualized at 300 V for 12 minutes. Sanger sequencing was carried out in accordance with the band information.

Table 1. Primer sequences

Primer name	Sequence of primer	Length
c.4030-F1	5'-ACTGGTCAGAGTAAAAGCCT-3'	734 bp
c.4030-R1	5'-ATTGCTCCTAATGCTATCCC-3'	
c.4030-F2	5'-GAACTGGTCAGAGTAAAAGCC-3'	737 bp
c.4030-R2	5'-TATTGCTCCTAATGCTATCCC-3'	
c.5000-F1	5'-GCTTCTGAGGGAGACAACCC-3'	618 bp
c.5000-R1	5'-GGCTCTCCAGTCAACTTCCC-3'	
c.5000-F2	5'-GCCCAGGAAGATCAGCTCTC-3'	764 bp
c.5000-R2	5'-CCTCCCTGACCCTTCTCTCA-3'	

SnapGene was used to verify the sequencing results.

3. Results

3.1. Imaging data and audiological assessments

According to the MRI of the inner ear (Figure 3), the proband had normal cochlear nerves in both ears. Thus, retrocochlear organic diseases such as cochlear nerve dysplasia were excluded. The average ASSR thresholds were 75 dB nHL in the right ear and 77.5 dB nHL in the left ear of the proband and 95 dB nHL in the right ear and 97.5 dB nHL in the left ear of her sister (II-1). According to the behavioral hearing test and PTA results, the proband had total hearing loss, and her sister had profound hearing loss. The air conduction and bone conduction results of the ABR showed that the proband did not elicit a reproducible wave on either side at 100 dB nHL and 50 dB nHL, respectively; both sisters had a

tympanogram result of "A". The proband was diagnosed with bilateral AN according to the results of DPOAE and CM tests (Figure 4). Patient II-1 was diagnosed with AN at 2 years of age, but DPOAE and CM were absent at 12 years of age (Figure 4). Therefore, II-1 was diagnosed with sensorineural hearing loss (SNHL). Her father suffered from high-frequency (4 kHz and 8 kHz) SNHL, which may have been caused by noise exposure, and her mother's hearing remained normal.

3.2. WES and variant analysis

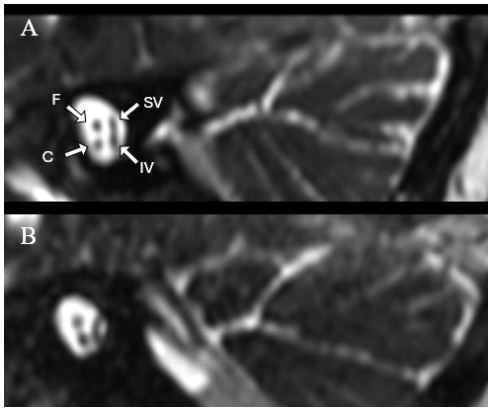

Figure 3. Oblique sagittal MRI of Patient II-2. (A) The distal slice shows the facial (F), superior vestibular (SV), inferior vestibular (IV) and cochlear (C) nerves of the right ear. **(B)** The distal slice shows the F, SV, IV, and C nerves of the left ear.

The average raw data of the sequencing samples was 11.04 Gb. The average effective data accounted for 98.93% of the data and the average Q20 was 97.85%. The average Q30 was 93.50%, and the average error rate was 0.03%. The average sequencing depth of the target region was 127.00×, and the average coverage was 99.33%. The variants were filtered as shown in Figure 2, and two mutation sites (c.4030C>T and c.5000C>A in the *OTOF* gene) were strongly related to the clinical phenotype. Both daughters presented compound heterozygosity of the nonsense mutation c.4030C>T (p.R1344X) and the missense mutation c.5000C>A (p.A1667D) in the *OTOF* gene. The c.5000C>A mutation has not been reported in HGMD, PubMed, or ClinVar; moreover, it is predicted to be harmful by SIFT, PolyPhen, and Mutation Taster and to be of uncertain significance according to the ACMG guidelines. The amino acid A1667 is conserved across multiple species (Figure 5). The pathogenicity data for the c.4030C>T mutation was collected from the databases. The patient's father was a heterozygous carrier of the c.5000C>A mutation, and the mother was a heterozygous carrier of the c.4030C>T mutation. The mutations were verified by Sanger sequencing (Figure 1B).

4. Discussion

The otoferlin protein encoded by the *OTOF* gene is an important part of the ribbon synapses, and its high

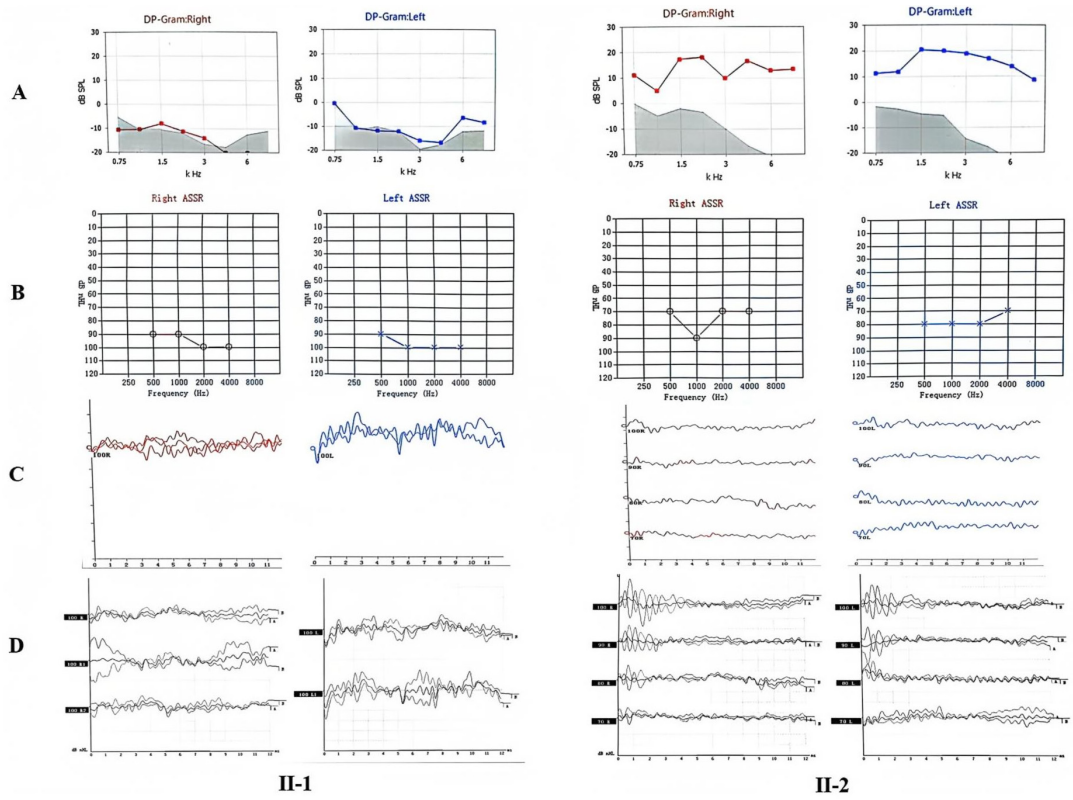

Figure 4. Audiologic tests of subjects II-1 and II-2. (A) DPOAE, distortion product otoacoustic emission; **(B)** ASSR, auditory steady-state response; **(C)** ABR, auditory brainstem response; **(D)** CM, cochlear microphonic potential.

Figure 5. Conservation analysis of the p.A1667 mutation site of otoferlin. The p.A1667 site is conserved in multiple species.

expression and proper localization are the basis for the accurate transmission of acoustic signals to auditory pathways (*25*). A mutation in the *OTOF* gene can cause structural changes and/or a decrease in otoferlin levels, weaken exocytosis of IHC ribbon synapses, and lead to different degrees of deafness (*11*). A total of 290 mutation sites in the *OTOF* gene have been reported through May 2024 (*26*), but most of them are sporadic and unique mutations.

In this study, the proband (II-2) exhibited clinical features typical of AN. Her sister (II-1) exhibited disappearance of DPOAE and CM. Severe auditory synaptopathy can eventually lead to the degeneration of IHCs and SGNs, which is why DPOAE/CM disappear in some patients during follow-up (*5*). Therefore, both of the sisters were diagnosed with AN. Two mutations in the *OTOF* gene were detected in their family by WES. The c.4030C>T mutation results in a premature stop codon by changing arginine to a terminator (p.R1344X), which leads to dysfunctional protein products that may be responsible for the pathogenesis of deafness. This mutation was previously reported in the deaf population in Pakistan and France (*27,28*), and this is the first time it has been identified in the Chinese population. The missense mutation c.5000C>A, located between the C2E and C2F domains, results in a single amino acid substitution of alanine to aspartic acid (p.A1667D) in a position that is in different species. The mutation c.5000C>A is assumed to be pathogenic because *i*) the affected individual in this family was diagnosed with AN, *ii*) the genotype and phenotype were coseparated, and *iii*) p.A1667 is conserved among different species.

Tang *et al.* developed a novel dual adeno-associated virus (AAV)-mediated gene therapy system based on the principles of protein trans-splicing that can reverse bilateral deafness in *Otof*$^{-/-}$ mice. The system effectively expressed exogenous mouse or human otoferlin and restored the release of synaptic vesicles in IHCs for a period after injection, providing a preferential clinical strategy for the treatment of *OTOF*-related AN (*15*). Since each IHC ribbon synapse is usually contacted by a single SGN, this degeneration adversely affects the effect of cochlear implantation (CI) or gene therapy (*29*). Thus, we suggested the patient (II-2) receive gene therapy at the ENT Institute and Department of Otorhinolaryngology, Eye & ENT Hospital, Fudan University, Shanghai, China. She was administered a single injection of AAV1-hOTOF into the cochlea through the round window at 3 years of age (*30*). Two months after the injection, the hearing level in both ears returned to 30 dB HL. The hearing recovery also indicates the missense mutation c.5000C>A is pathogenic.

5. Conclusion

We identified two disease-causing mutations in the *OTOF* gene in a Chinese family with AN by WES, which indicated that WES is an effective approach for identifying the hereditary characteristics of AN. The identification of these two mutations expands the known mutation spectrum of the *OTOF* gene in the Chinese population and provides a basis for gene therapy.

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Original Article

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Lysine succinylation analysis reveals the effect of *Sirt5* on synovial fibroblasts in rheumatoid arthritis patients

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SUMMARY Rheumatoid arthritis (RA) is an autoimmune disease with complex etiology, and its pathological mechanism remains unclear. Our aim was to explore the effect of protein succinylation on RA by silencing *Sirt5*, sequencing succinylated proteins, and analyzing the sequencing results to identify potential biomarkers. We wanted to gain a clearer understanding of RA pathogenesis, quantitative assessment of succinylated proteins in Fibroblast-like synoviocytes (FLS) from RA patients using liquid chromatography- tandem mass spectrometry and enrichment analysis investigated using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG). A total of 679 proteins and 2,471 lysine succinylation sites were found in RA patients, and 436 differentially expressed proteins and 1,548 differentially expressed succinylation sites were identified. Among them, 48 succinylation sites were upregulated in 38 proteins and 144 succinylation sites were downregulated in 82 proteins. Bioinformatics showed that succinylated proteins were significantly enriched in amino and fatty acid metabolisms. Results indicated that *Sirt5* can affect various biological processes involved in RA FLSs, and succinylation caused by silencing *Sirt5* plays a major role in RA progression. This study provides further understanding of RA pathogenesis and may facilitate searching for potential RA biomarkers.

Keywords rheumatoid arthritis, synovial fibroblasts, *Sirt5*, succinylation

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation in multiple joints. Its pathological features are synovitis and pannus formation, resulting in joint damage, cartilage destruction, bone erosion, and eventually joint deformity and loss of function (*1*). Genetic and environmental factors are involved in the occurrence and development of RA (*2*). Because of the complexity of its pathological factors and pathogenesis, it has not yet been fully clarified. Therefore, understanding the molecular mechanism of RA occurrence and development is very important for RA treatment.

Post-translational modification of proteins can alter the structure and function of proteins and plays a major role in the occurrence and development of diseases (*3*). Lysine is a frequent target of modification among amino acid residues in proteins. In a recent study, lysine succinylation was defined as the transfer of a succinyl group to a lysine residue, which is a newly discovered post-translational modification of proteins (*4*).

Sirt5 is a member of the Sirtuin family and influences the occurrence and development of many diseases. *Sirt5* is involved in the regulation of various cellular processes (*5*), such as reactive oxygen species defense (*5*), fatty acid metabolism (*6*), and apoptosis (*7*). Expression of *Sirt5* is low in RA patients and affects RA development by inhibiting the secretion of proinflammatory factors (*8*). However, the precise mechanism by which *Sirt5* affects RA remains unclear. Therefore, targeting *Sirt5* provides a possibility for treating RA.

In this study, we identified 436 differentially expressed proteins and 1,548 differential succinylation sites after silencing *Sirt5*, followed by protein extraction, affinity enrichment, and liquid chromatography-tandem mass spectrometry (LC-MS/MS). These succinylated proteins were involved in various biological functions and cellular processes. Our study may facilitate understanding the regulatory role of *Sirt5*-mediated lysine succinylation in RA.

2. Materials and Methods

2.1. Synovial tissue collection

Synovial tissue was collected from RA patients during knee replacement surgery. All patients met the RA diagnostic criteria of the American College of Rheumatology. This study was approved by the Medical Ethics Committee of the Institutional Review Board of Shandong Medicinal Biotechnology Center (SMBC-2020-08). Synovial tissue was collected at Shandong Provincial Hospital (Ji'nan, Shandong). Written informed consent was provided by the patients.

2.2. Preparation and culture of synovial fibroblasts from RA patients

The synovial tissue was cut into pieces, digested with type II and III collagenases (Sigma-Aldrich, St. Louis, MO, USA), and cultured at 37°C for 4–5 h. The digestion was terminated by incubation in Dulbecco's modified Eagle's medium DMEM (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA, USA) for 24 h. After washing with phosphate-buffered saline (PBS) (Solabio, Beijing, China), the cells were cultured in 10% fetal bovine serum-containing medium.

2.3. SiRNA-mediated silencing of *Sirt5* expression in synovial fibroblasts

After screening the silencing efficiency of *Sirt5*-targeting siRNAs designed and produced by Gemma Company (Gemma, Shanghai, China), the siRNA with the highest silencing efficiency was selected for the following experiment. The SiSIRT5 sequences were 5′-GGAGAUCCAUGGUAGCUUATT-3′ and 5′-UAAGCUACCAUGGAUCUCCTT-3′. HiPerFect transfection reagent (Qiagen) was used for transfection in accordance with the manufacturer's instructions. SiNC was provided by Qiagen. After incubation for 48 h, cells were collected and stored in liquid nitrogen until analysis.

2.4. Protein extraction and pancreatic enzymolysis

Samples were mixed with a four-fold volume of cracking buffer (8 mol/L urea, 1% protease inhibitor, 3 μmol/L TSA, and 50 mmol/L NAM) for ultrasonication. After centrifuging at 4°C for 10 min at 12,000 × g, the supernatant was transferred to a new centrifuge tube, and the protein concentration was determined using a BCA protein concentration determination kit. The proteins in each sample were enzymolized in the same amount, and then the volume was adjusted to the same volume as the lysate. Trichloroacetic acid (TCA) (Sigma-Aldrich, St. Louis, MO, USA) was slowly added to a final concentration of 20%, and the sample was precipitated at 4°C for 2 h. After centrifugation at 4,500 × g for 5 min, the precipitate was washed with pre-cooled acetone two to three times. After drying and precipitation, tetraethylammonium bromide (TEAB) (Sigma-Aldrich, St. Louis, MO, USA) was added to a final concentration of 200 mmol/L. The precipitate was dispersed by ultrasonication, and trypsin (Promega Corporation, Fitchburg, Wisconsin, United States) was added at a ratio of 1:50 (protease:protein, m/m), and enzymatic hydrolysis was carried out overnight. Dithiothreitol (DTT) (Sigma-Aldrich, St. Louis, MO, USA) was added to a final concentration of 5 mmol/L and reduced at 56°C for 30 min. Then, iodoacetamide (IAM) (Sigma-Aldrich, St. Louis, MO, USA) was added to a final concentration of 11 mmol/L, followed by incubation at room temperature for 15 min while protected from light.

2.5. Enrichment by Ksu modification

Proteins were dissolved in IP buffer (100 mmol/L NaCl, 1 mmol/L EDTA, 50 mmol/L Tris-HCl, and 0.5% NP-40, pH 8.0). The supernatant was transferred to pre-washed resin (antibody resin catalog number PTM402, PTM Bio, Hangzhou, China) and placed on a rotating mixer at 4°C overnight. Then, the resin was washed with IP buffer four times and then with deionized water twice. Finally, the resin-bound peptides were eluted using 0.1% trifluoroacetic acid eluent (TFA)(Sigma-Aldrich, St. Louis, MO, USA), the eluate was eluted three times, and the eluent was collected and dried using vacuum freezing. After desalting using C18 ZipTips (Millipore) in accordance with the manufacturer's instructions, the sample was dried again using vacuum freezing for LC-MS analysis.

2.6. Liquid chromatography-mass spectrometry

Peptides were dissolved in mobile phase A and separated in a NanoElute (Bruker, Germany) ultra-high performance liquid phase system. Mobile phase A was an aqueous solution containing 0.1% formic acid and 2% acetonitrile. Mobile phase B was an acetonitrile-aqueous solution containing 0.1% formic acid. The liquid phase gradient settings were: 0–40 min, 7–24% B; 40–52 min, 24–32% B; 52–56 min, 32–80% B; 56–60 min, 80% B. The flow rate was maintained at 450 nL/min. Peptides were separated in an ultra-high performance liquid phase system, injected into the capillary ion source for ionization, and then analyzed by timsTOF Pro 2 mass spectrometry. The ion source voltage was set to 1.5 kV. The parent ion of the peptide segment and its secondary fragments were detected and analyzed using high-resolution TOF. The secondary mass spectrometry scan range was set to 100–1700. The data acquisition mode used the parallel cumulative serial fragmentation

(PASEF) mode. A secondary spectrum with a charge number of parent ions in the range of 0–5 was collected in PASEF mode 10 times after primary mass spectrum collection. The dynamic exclusion time of the series mass spectrometry scanning was set to 24 s to avoid repeated scanning of parent ions.

2.7. Database searching

Secondary mass spectrum data were retrieved using Maxquant (v1.6.15.0), and the enzyme digestion method was set to Trypsin/P. The number of missing cuts was set to 4. The minimum length of the peptide was set to 7 amino acid residues. The maximum number of peptide modifications was set to 5. The mass error tolerance for primary parent ions was set to 20 ppm for first and main searches, and secondary fragment ions. The false discovery rate (FDR) for protein, peptide and modification site identification was set to 1%.

2.8. Bioinformatics data analysis

Gene Ontology (GO) was performed for functional analysis of differentially expressed proteins, including biological processes, cell localization and molecular function. Pathway enrichment analysis of differential genes was performed by Kyoto Encyclopedia of Genes and Genomes (KEGG). Motif analysis of modification sites employed the MoMo analysis tool based on the MotiF-X algorithm. After database comparison with the STRING protein interaction network, the R package visNetwork tool was used for visual analysis of differentially expressed proteins. The interaction relationship of differentially modified proteins was extracted using a confidence score of > 0.7 (high confidence).

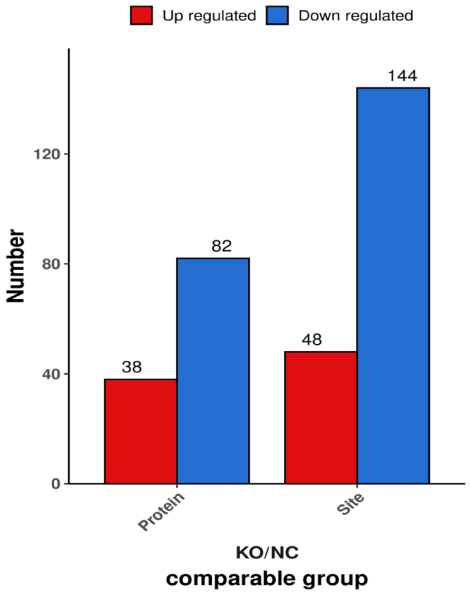


Figure 1. Expression of differentially modified proteins and modification sites.

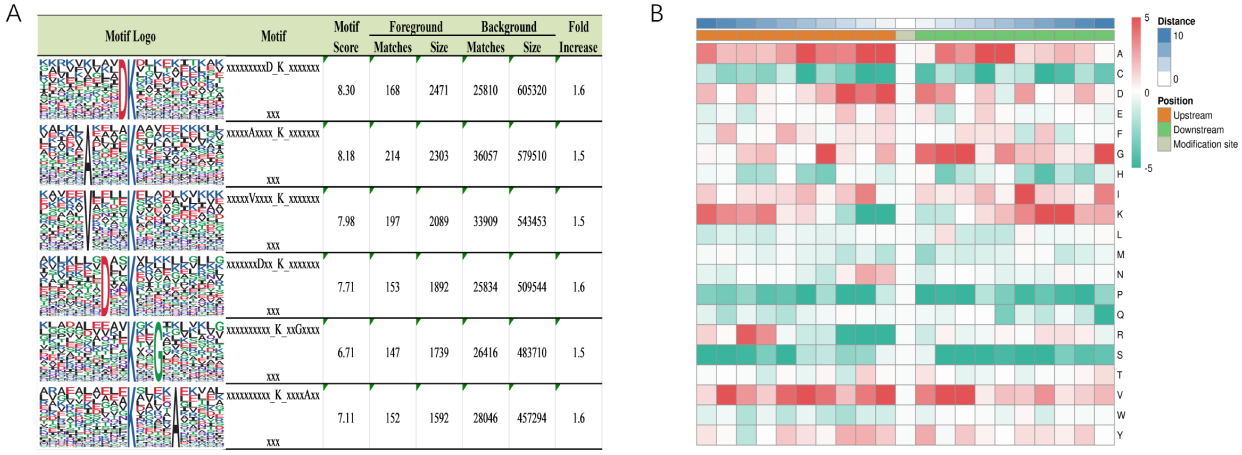
3. Results

3.1. Quantitative modification of Ksu in siSIRT5 and siNC groups

A total of 436 differentially expressed proteins were identified by LC-MS/MS in siSIRT5 and siNC groups with 1,548 lysine succinylation sites. Among them, 38 upregulated proteins had 48 succinylation sites and 82 downregulated proteins had 144 succinylation sites(Figure 1). The samples were set as three group repeats. At $p < 0.05$, the differential expression level was > 1.5 as the significantly upregulated change threshold and < 0.67 as the significantly downregulated change threshold.

3.2. Ksu motif analysis

To clarify the specific lysine succinylation sequence of



Motif Logo	Motif	Motif Score	Foreground Matches	Foreground Size	Background Matches	Background Size	Fold Increase
	xxxxxxxxxD_K_xxxxxx	8.30	168	2471	25810	605320	1.6
	xxxxxAxxxx_K_xxxxxx	8.18	214	2303	36057	579510	1.5
	xxxxxVxxxx_K_xxxxxx	7.98	197	2089	33909	543453	1.5
	xxxxxxxDxx_K_xxxxxx	7.71	153	1892	25834	509544	1.6
	xxxxxxxxx_K_xxGxxxx	6.71	147	1739	26416	483710	1.5
	xxxxxxxxx_K_xxxxAxx	7.11	152	1592	28046	457294	1.6

Figure 2. Motif analysis of succinylated peptides. (A) Sequence markers of succinylation motifs; (B) Heat map of amino acid frequency near the succinylation site.

SIRT5 in rheumatoid joints, we conducted motif analysis of amino acids at succinylation sites around lysine. The lysine succinylation sites were mainly enriched in DK, A****K, V****K, D**K, K**G, and K****A (Figure 2A). By evaluating the frequency of amino acids at the succinylation sites, we found highly site-specific alanine (A), aspartic acid (D), and valine (V) at multiple sites (-1, -2, -3, -4, and -5), but different degrees of deletion at corresponding sites upstream (Figure 2B). Cysteine (C), proline (P), and serine (S) were rarely present, and glycine (G) and leucine (L) were highly enriched at +2, but absent at -2, whereas aspartate (N) was only highly present at -1 and -2, and lysine was highly present at ±7, ±8, ±9, and ±10.

3.3. Enrichment analysis of differentially expressed succinylated proteins

To elucidate the function of differentially regulated lysine succinylation genes after *Sirt5* silencing, the GO database was used for functional analysis and the KEGG database was used for pathway analysis. The GO database includes three major components: cells, biological processes, and molecular functions. For cell components, expression was mainly upregulated in mitochondria and downregulated in the cytoplasm, nucleus, and chromosomes (Figure 3A,3B). For molecular functions, enzyme binding, unfolded protein binding, and cytoskeletal protein binding were downregulated

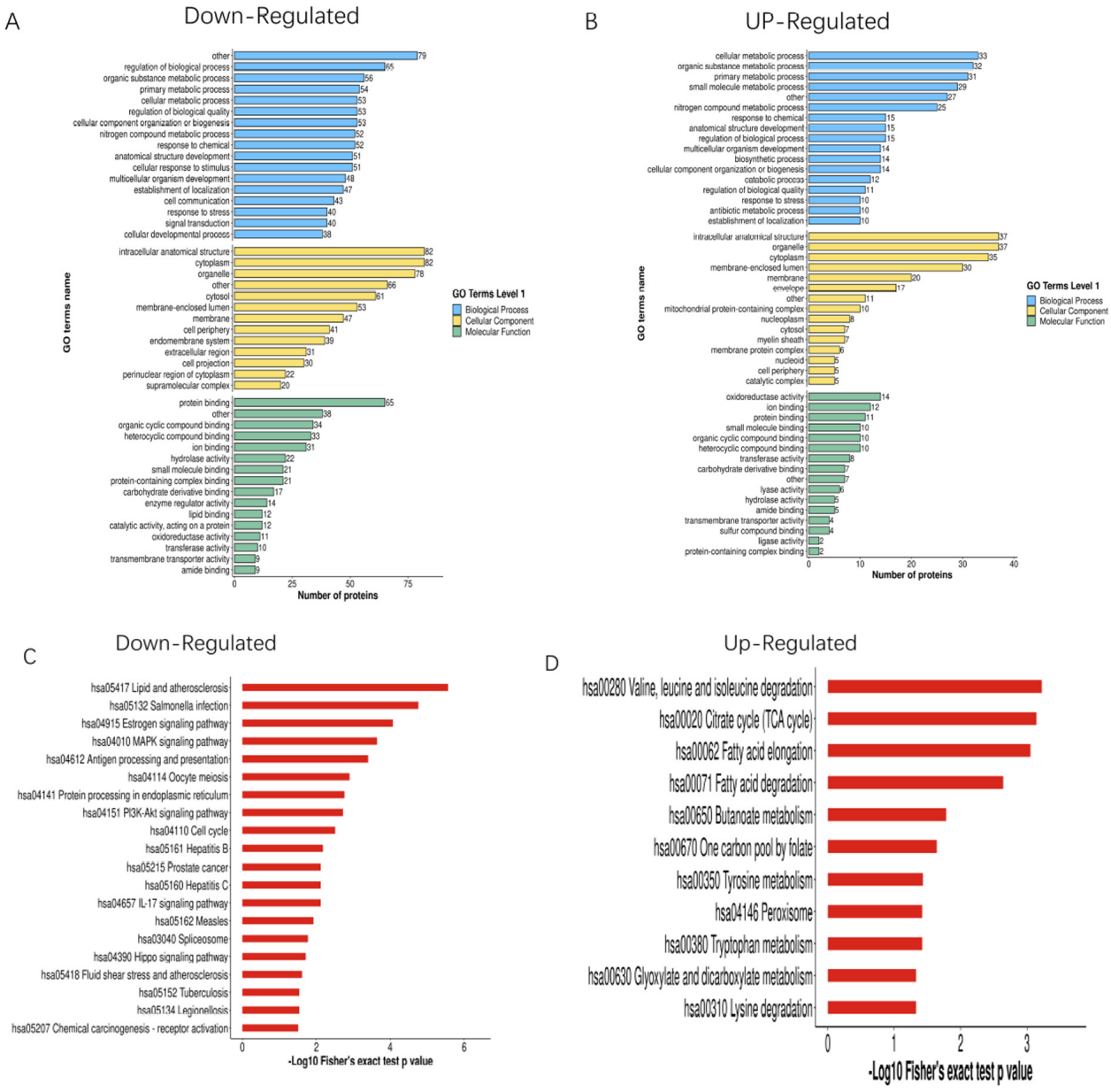


Figure 3. Functional analysis of lysine succinylation group in synovial fibroblasts of rheumatoid arthritis. (A) up- and (B) downregulated succinylated proteins were examined by GO functional enrichment; (C) up- and (D) downregulated succinylated proteins were examined by KEGG pathway analysis.

(Figure 3A), fatty acid acyl-CoA and fatty acid derivatives were bound, and acyl-CoA dehydrogenase activity was significantly upregulated (Figure 3B). For biological processes, metabolic processes, such as citrate metabolism, tricarboxylic acid metabolism, aerobic respiration, and fatty acid oxidation, were significantly upregulated (Figure 3B), while biological processes, such as protein localization in the nucleus, positive regulation of organelles, and regulation of cell cycle phase transition, were significantly downregulated (Figure 3A).

KEGG signaling pathway enrichment analysis showed that related metabolic processes, such as branched chain amino acids, the tricarboxylic acid (TCA) cycle, fatty acid extension and degradation, and one-carbon metabolism, were significantly enriched (Figure 3D), and the estrogen signaling pathway, the Mitogen-Activated Protein Kinase (MAPK) signaling pathway, endoplasmic reticulum protein processing, and cell cycle signaling pathway were significantly downregulated (Figure 3C).

3.4. Differentially expressed protein network interaction analysis

Protein–protein interaction (PPI) network analysis of succinylated groups was conducted. Upregulated genes in the PPI network were mainly mitochondrion-related metabolic enzymes, while the downregulated proteins were closely related to cytoskeletal assembly, protein synthesis, endoplasmic reticulum stress, and other processes. Expression of DUT, SHMT2 (nucleotide synthesis related), NNT, SDHA (mitochondrial respiratory chain related), HMGCL, IVD (leucine catabolic metabolism related), ACAD9, UQCRB (oxidative phosphorylation), IDH2, and CS (tricarboxylic acid cycle) were obvious among upregulated proteins (Figure 4). Thus, it is highly likely that *Sirt5* affects RA progression by affecting succinylation of these molecules.

4. Discussion

In this study, novel label-free proteomic quantitative technology was used to quantitatively analyze RA FLSs, and the potential mechanism of succinylation in the occurrence and development of rheumatoid arthritis was investigated by quantitative succinylation analysis after *Sirt5* silencing. Compared with the treatment group, 436 differentially expressed proteins with 1,548 succinylation sites were found. By GO and KEGG analysis of different succinylated proteins, it was found that high expression of succinylated proteins was mainly associated with mitochondria, suggesting that succinylation affects mitochondrial function. In addition, KEGG pathway enrichment analysis showed that the up-regulated pathway was mainly concentrated in mitochondria-related metabolism, such as branched-chain amino acid degradation, TCA cycle, fatty acid metabolism, *etc.* According to previous studies (*9*), high succinylation in brain injury after cerebral hemorrhage mainly affects

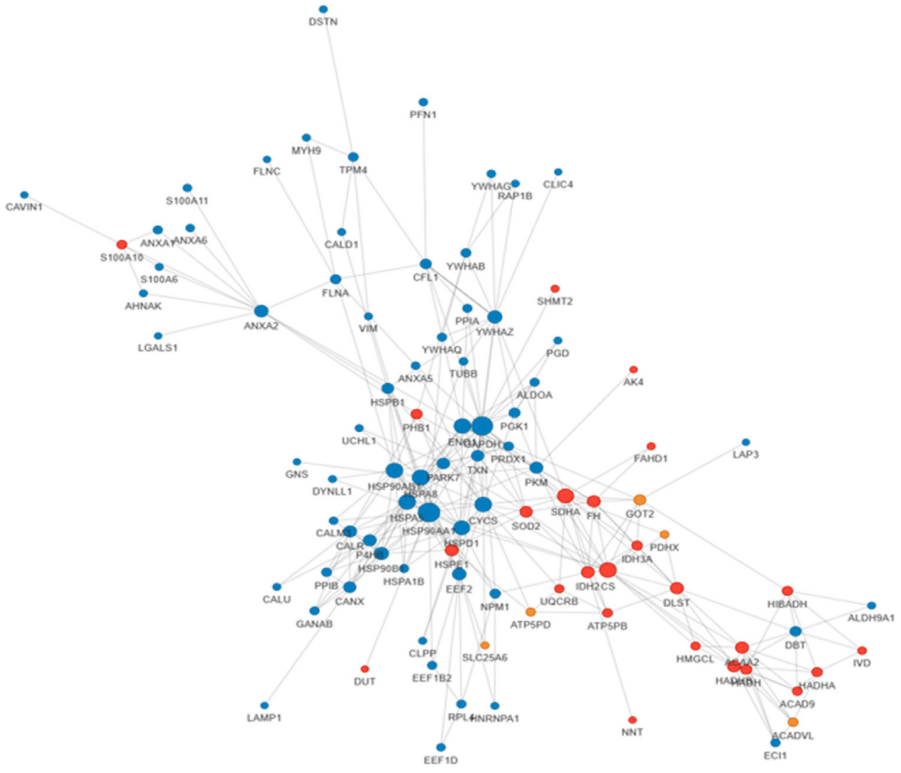


Figure 4. Protein–protein interaction network of succinylated proteins in synovial fibroblasts of rheumatoid arthritis.

fatty acid metabolism, and most of the subcellular localization of succinylation occurs in mitochondria. This suggests that succinylation affects disease development primarily by affecting mitochondria-related metabolism. Metabolic disorders involve almost all the pathogenesis stages of RA, and the humoral metabolome related to RA indicates that the disorder of RA metabolism may be related to maintenance of the inflammatory environment, which leads to an increased demand for biological energy and biosynthesis (*10*). This may require changes in mitochondrial metabolic pathways to meet the chronic inflammation of RA. The TCA cycle is the main productive pathway in mitochondria, and the raw materials required for the tricarboxylic acid cycle can be obtained through the degradation of branched-chain amino acids, which may be the reason for elevation of the two metabolic pathways after the succinylation of RA. Mitochondria play an important role in maintaining the normal life activities of cells. Dysfunction will lead to the release of inflammatory mediators and the aggregation of inflammatory cells, aggravate the inflammation of RA, inhibit the apoptosis of synovial cells, and promote the invasion of synovial cells (*11*).

Sirt5 is a desuccinylase. According to relevant literature reports, the expression of sirt5 is down-regulated in activated macrophages, which indicates that the decreased expression of *sirt5* may be related to the increase of RA and play a protective role in RA (*12*). Therefore, the molecules that are up-regulated in the succinylation data after the knockdown of sirt5 may play a role in RA. We will continue to study these molecules. Through PPI analysis, it was shown that upregulated proteins such as HSPE1, DUT, NNT, HMGCL, IDH2, IVD, ACAD9, UQCRB, SHMT2, SDHA, HADHA, HADHB and CS were all metabolic enzymes related to mitochondria. SHMT2, a binding protein of pyridoxal phosphate, plays an important role in catalytic catabolism of serine and promotes cancer cell proliferation (*13*). Succinylation activity of SHMT2 is regulated by *Sirt5* desuccinylase, and an increase in *Sirt5* expression downregulates SHMT2 enzymatic activity, thereby inhibiting cancer cell proliferation (*14*). IDH2 is a reduced nicotinamide adenine nucleotide phosphate (NADPH)-dependent enzyme, which catalyzes the conversion of isocitric acid into α-ketoglutaric acid through oxidative decarboxylation. NAPDH produced in this process is a cofactor, which maintains cell oxidation homeostasis, plays a role in maintaining cell REDOX function in cancer cell metabolism, and promotes tumor cell invasion by affecting mitochondrial dynamics (*15*). According to previous studies, sirt5 can promote IDH2 desuccinylation, reduce oxidative stress of cardiomyocytes, and maintain cell REDOX homeostasis (*16*). CS is a rate-limiting enzyme in the tricarboxylic acid cycle, and the enzyme activity of CS is regulated by succinylation. Previous studies have confirmed that *Sirt5* can desuccinylate CS at K393 and K395, thus affecting the proliferation and migration of colon cancer cells (*17*). SDHA is mainly involved in the TCA cycle and oxidative phosphorylation. Studies have shown that SDHA interacts with sirt5 to desuccinylate SDHA, and the desuccinylation of SDHA will weaken the interaction with SDH5 and promote the proliferation of renal cancer cells (*18*). Therefore, targeting these metabolism-related molecules has potential for research in rheumatoid arthritis.

The pathogenesis of rheumatoid arthritis is extremely complex. However, abnormal energy metabolism plays a major role in the process of rheumatoid arthritis (*19*). Mitochondria are the main sites of energy supply and participate in various metabolic pathways. They also play an important role in maintaining the internal environment of synovial cells (*20*). Studies of molecular interactions and functions in mitochondria contribute to understanding the pathogenesis of rheumatoid arthritis, and proteins with differential succinylation are likely to be potential biomarkers of rheumatoid arthritis.

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

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Reprogramming the future: Capitalizing on *in vitro* embryo culture by advancing stem cell technologies in the fight against rare genetic disorders

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SUMMARY Capitalizing on breakthroughs in reproductive genetics, the utilization of *in vitro* embryo culture and stem cell technologies heralds a transformative era in addressing global challenges posed by rare genetic diseases. These cutting-edge practices illuminate the intricacies of early human development, elucidate the mechanisms behind rare diseases, and guide the development of potential therapies. Balancing this remarkable innovation with necessary ethical considerations, these technologies have the potential to revolutionize the trajectory of rare genetic disorders, transforming the landscape of diagnosis, treatment, and genetic counseling while offering renewed hope for affected individuals and families worldwide.

Keywords embryo engineering, reproductive genetics, genetic disorders, stem cell therapies, ethical balance

Despite continuous advances in embryo engineering and reproductive genetics, the persistence of birth defects remains a significant challenge both nationally and globally. These defects contribute to a substantial portion of the population affected by rare genetic diseases. In fact, more than 80% of these rare disorders have a genetic basis since they are primarily caused by gene mutations (*1*). Among the 6,000+ recognized rare diseases worldwide, nearly half manifest at birth or during childhood, yet only a small fraction of these disorders have effective treatments, as evinced by the fact that specific therapies are currently available for less than 6% of these diseases (*2*). A previous study indicated that there are 16.8 million patients with rare diseases, but given China's population of 1.4 billion, this number is significantly underestimated (*3*). To address this issue, there is a growing need to rely more heavily on assisted reproductive technologies and prenatal diagnosis techniques. These methods play a crucial role in identifying and preventing the transmission of birth defects associated with rare diseases. Assisted reproduction technologies, such as *in vitro* fertilization (IVF) and preimplantation genetic testing (PGT), can help identify embryos carrying specific genetic mutations linked to rare diseases. By selectively transferring unaffected embryos into the uterus, the risk of passing on these genetic diseases to offspring can be significantly reduced (*4*). Indeed, the quality of embryos plays a crucial role in ensuring a healthy pregnancy. As a result, embryo culture has emerged as a significant and challenging research topic in the field of medical research.

Within the field of technology to prevent birth defects, the utilization of *in vitro* embryo culture models is vital. The process of embryogenesis, which involves the development of morphology and function, is remarkably complex and regulated at multiple levels (*5*). By experimentally manipulating and observing embryo development in *in vitro* cultures, we can enhance our understanding of the early stages of human embryogenesis. This, in turn, facilitates research on various aspects, including the mechanisms underlying early human genetic diseases, structural modifications of rare disease genes, and the screening of therapeutics. The use of *in vitro* embryo culture models provides valuable insights into the intricate processes of embryogenesis. It enables researchers to study and comprehend the initial stages of human development, shining light on the underlying mechanisms of rare genetic diseases (*6*). This knowledge can aid in

identifying and understanding structural modifications in genes responsible for rare diseases (*7*). In addition, *in vitro* embryo culture models offer a platform to screen and evaluate potential therapeutics, potentially leading to the discovery of new treatments for these conditions.

Overall, the use of *in vitro* embryo culture models represents a significant advance in technology to prevent birth defects. It contributes to our understanding of early human development and enables critical research on rare genetic diseases, gene modifications, and the screening of potential treatments (*8*). Ultimately, the meticulous nature of embryo *in vitro* culture has revolutionized how we approach reproductive medicine, and especially in the context of rare diseases. Through procedures like preimplantation genetic testing, we can detect and mitigate the risk of passing on these disorders to future generations (*9*). By combining *in vitro* fertilization with PGT, couples with known genetic disorders can make informed decisions about which embryos to implant, greatly reducing the likelihood of having a child affected by a rare disease.

In addition to PGT, embryo *in vitro* culture also allows for other advances in the understanding and management of rare diseases. Researchers can study the development of embryos in a controlled laboratory environment, providing insights into the early stages of human embryogenesis and the mechanisms underlying rare genetic diseases. This knowledge can contribute to the development of more effective treatments. *In vitro* embryo culture is a powerful tool that empowers couples to make informed choices, reduces the reproductive risks associated with rare diseases, and enhances our overall understanding of these conditions (*10*). As research continues to advance in this field, it holds significant promise for improving the outcomes and quality of life for individuals affected by rare genetic diseases and interventions for those individuals (Figure 1).

In vitro culture does offer researchers valuable insights into early human development and can contribute to our understanding of rare genetic diseases. The ability to study embryos in a controlled laboratory environment has resulted in important discoveries, such as those related to Rett syndrome and the *MECP2* gene (*11*). However, the ethical concerns surrounding embryo *in vitro* culture need to be acknowledged. The manipulation and potential discarding of human embryos raise complex ethical questions about the beginning of life, the moral status of the embryo, and the ethical implications of conducting research on embryos. These ethical concerns have led to ongoing debates and discussions in the scientific and wider communities. Various ethical guidelines, regulations, and informed consent processes are in place to ensure that embryo research is conducted responsibly and within the bounds of ethical considerations (*12,13*). Balancing scientific progress with ethical principles is important in order to promoting responsible research practices and public trust in the field of *in vitro* embryo culture.

The future of *in vitro* stem cell culture holds immense promise, particularly in the realm of rare diseases. The unique ability of stem cells to differentiate into various cell types offers tremendous potential for developing novel therapeutic approaches, and especially for diseases for which there are currently no effective treatments (*14*). These cells can serve as invaluable tools in modeling disease pathogenesis and conducting drug screening. One remarkable example is the use of induced pluripotent stem cells (iPSCs), which can be generated from a patient's own cells and reprogrammed into a stem cell-like state (*15*). These iPSCs can then

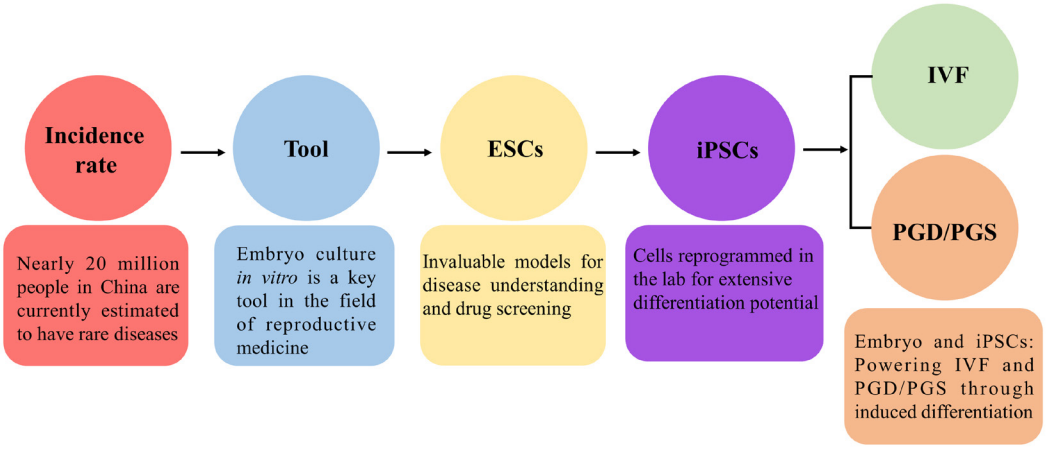

Figure 1. Visualizing the intersection: rare diseases, stem cells, and assisted reproductive technologies. This diagram encapsulates the prevalence of rare diseases in China, the importance of *in vitro* embryo culture, the utility of embryonic and induced pluripotent stem cells in disease modeling and drug screening, and the potential of combining these with assisted reproductive technologies such as IVF and preimplantation genetic testing. The interconnected circles highlight the synergy of these elements in addressing rare genetic disorders in the field of reproductive medicine.

be differentiated into the specific cell type affected by the rare disease. This groundbreaking technology has already been used to successfully model numerous rare diseases in laboratory settings, providing unprecedented insights into the underlying cellular and molecular mechanisms at play. Moreover, stem cell research opens up possibilities for regenerative therapies. By utilizing stem cells from patients, damaged tissues could potentially be replaced with healthy cells, leading to a transformative approach in treating rare diseases (*16*).

As research in *in vitro* stem cell culture advances, ethical considerations need to continue to be made, appropriate regulations need to be enacted, and informed consent processes need to be followed to maintain public trust. These combined efforts will undoubtedly contribute to the continued progress and potential of stem cell research in addressing rare diseases. The use of *in vitro* stem cell culture for the targeted treatment of rare diseases holds immense potential, but it also presents several significant challenges that need to be addressed. While regenerative medicine offers great promise, much research still needs to be conducted to fully understand how to reliably guide stem cells in their differentiation process and to ensure their safe integration into the patient's body. Overcoming these challenges will require continued scientific advances and rigorous testing to ensure the efficacy and safety of stem cell therapies.

Ethical considerations and regulatory frameworks also play a vital role in the use of stem cell research (*17*). The use of specific types of stem cells may raise ethical concerns, and careful consideration must be given to ensure that research is conducted within ethical boundaries and adheres to established regulations and guidelines (*18*). Another obstacle to widespread implementation is the complexity and cost associated with creating and differentiating iPSCs. These complexities may limit accessibility and hinder the potential reach of emerging treatments, particularly in settings with limited resources. To overcome this, efforts are underway to develop more efficient and cost-effective techniques for generating and differentiating stem cells.

In conclusion, the utilization of *in vitro* embryo culture in the fight against rare genetic disorders holds immense promise. By capitalizing on pluripotent stem cells and gene therapy techniques, this innovative approach has the potential to revolutionize the diagnosis, modeling, and treatment of these conditions (*19*). Ongoing ethical discussions, combined with efforts to improve accessibility, will play a crucial role in shaping the future of *in vitro* embryo culture and its impact on rare genetic disorders.

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Critical issue in the identification of Down syndrome and its problems in Central Java, Indonesia: The fact of needing health care and better management

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SUMMARY We conducted a cross-sectional study to describe the health care problems of children with Down syndrome in Central Java, Indonesia. A total of 162 children (81 boys, 81 girls) with Down syndrome were included. Congenital heart defects and hypothyroidism were found in about 50%, followed by vision and hearing problems in 27.7% and 17.3%, respectively. Almost half of cases were diagnosed after the first month of age. Advanced maternal age was identified in more than 50%, and less than 10% was based on karyotype analysis. This study describes the essential issues such as critical co-morbidities, delayed diagnosis, advanced maternal age, and lack of (accessibility to) genetic testing facilities; thus, better health care and management is needed.

Keywords delayed diagnosis, Down syndrome, genetic facilities, limited accessibility, Indonesia

1. Introduction

Down syndrome, also called trisomy 21, is the most common numerical chromosomal aberration found in live-born infants. Down syndrome is characterized by peculiar physical features such as hypotonia, epicanthic folds, up-slanting palpebral fissures, protruding tongue, simian creases, sandal gaps, and intellectual disability. The prevalence of Down syndrome was 1.7 to 2.5 in 1,000 live births, and increases with maternal age. In Indonesia, the prevalence of Down syndrome increased from 0.12% in 2010 to 0.21% in 2018 (*1*).

The frequency of congenital heart disease-associated Down syndrome was about 60%, which leads to early infant death (*2*). Early diagnosis and detection of treatable co-occurring medical conditions, such as hypothyroidism, congenital heart disease, and hearing problems, may minimize the complications, optimize the treatment, and prevent further irreversible conditions. In order to reach maximum beneficial outcomes, recognition of Down syndrome's typical characteristics, and early intervention, should begin shortly after birth (*3*).

Indonesia is the world's fourth-most populous country, with an estimated population of 275 million spread over 17,500 islands. A recently introduced national health insurance system covered almost 75% (203 million) of inhabitants (*4*), and is expected to improve equity and access to health care services, especially in remote areas/islands. However, prioritizing communicable diseases leads to limited access to genetic diseases, including Down syndrome (*5*). In fact, the quality of maternal health care services varies greatly between countries, and in developing countries such as Indonesia, where human resources and care facilities are scarce, this results in inequality of access to maternity and neonatal services (*6*). In developed countries, prenatal screening and diagnosis followed by pregnancy termination were recommended and offered, especially for advanced maternal age (*7*), while in Indonesia, the availability of prenatal diagnosis and termination of pregnancy are extremely low. Therefore, neonatal care, including early diagnosis, prompt intervention, and continued medical issues surveillance, is essential to improve the survival rate and quality of maternal and neonatal health.

Down syndrome based on clinical features, with and without chromosomal testing, is responsible for a significant proportion of infant and childhood

mortality in developed and developing countries but also contributes to chronic health problems and most lifelong disabilities (*8*). Among Down syndrome pregnancies, 63% resulted in a live birth (*9*). Indonesia has the largest Muslim population and religious belief plays an important role in daily life. In addition, the pregnancy termination policy is very strict and mostly offered only for maternity-related health problems; thus, termination in the case of an affected baby is not considered (*10*). Previous studies found that most Down syndrome was suspected on the day of birth, but delayed and appropriate diagnoses were reported (*11*). Therefore, this study aims to describe the healthcare problems in children with Down syndrome. This cross-sectional study was conducted at the Pediatric outpatient clinic of three referral hospitals in Central Java. This study was approved by the Ethics Committee (100/EC/KEPK/FK-UNDIP/VI/2020) and written consent was obtained from all respondents. Down syndrome diagnosis based on the six clinical features, that is, epicanthic folds, hypotonia, up-slanted palpebral fissures, protruding tongue, simian crease, and sandal gap were used for clinical diagnosis of Down syndrome (*12*), with and without chromosomal testing.

2. Clinical data

A total of 162 children with Down syndrome were included in this study, 81 cases were boys and 81 cases were girls, the mean age was 1.75 ± 2.04 in boys and 1.87 ± 2.26 in girls. Subjects were from the middle socio-economic level in almost two-thirds of cases (73.5%), and more than half (52.5%) were from advanced-age mothers at childbirth. Full-term gestational age was the majority of cases in both sexes (77.8%); and pre-term gestational age was found in 21.6%. Vaginal mode of delivery was found in 59.9%, while the remaining cases were born by caesarean section. The obstetrician was the most predominant birth assistant in this study (53.7%), followed by the midwifery-assisted delivery mode in 44.4%. More than 70% were born with normal birth weight; however, 30% were born with low birth weight (Table 1).

Based on Devlin and Morrison (*12*), hypotonia was found to be similarly prevalent in boys (88.8%) and girls (84.0%). Epicanthal fold was found in 96.3% of all cases and up slanted palpebral fissures was 95.1% in both boys and girls. The protruding tongue was observed in 64.2% of boys and 75.3% of girls. Dysmorphism in limbs was found higher in feet, and sandal gap occurrence was found to be similar in boys (91.4%) and girls (88.9%), followed by a single palmar crease observed in 77.8% of both boys and girls. Two characteristics frequently found in addition to Devlin and Morrison clinical criteria, which is flat nasal bridge was found at a higher rate in boys (92.6%) and girls (95.1%). Fifth finger clinodactyly was discovered in 86.4% of boys, and 84.0% of girls Table 2).

Congenital heart diseases and hypothyroidism were found in approximately half of the cases. Hearing problems were found in almost one in five boys (19.8%) and somewhat lower in girls (14.8%), while vision problems were found to be higher in girls (32.1%) compared to boys (23.5%) (Figure 1A).

Table 1. Demographic characteristics

Characteristics	Boys (*n*, %)	Girls (*n*, %)	Total (*n*, %)
Number	81 (50%)	81 (50%)	162 (100%)
Age, years (Mean ± SD)	1.75 ± 2.04	1.87 ± 2.26	-
Parental Socio-economic Status			
< 2,000,000 IDR	22 (%)	21 (%)	43 (26.5%)
≥ 2,000,000 IDR	59 (%)	60 (%)	119 (73.5%)
Maternal Age			
≤ 35 years old	40 (49.4%)	37 (45.7%)	77 (47.5%)
> 35 years old	41 (50.6%)	44 (54.3%)	85 (52.5%)
Gestational Age			
Pre-term (< 39 weeks)	15 (18.5%)	20 (24.7%)	35 (21.6%)
Full-term (39–40 weeks)	66 (81.5%)	60 (74.1%)	126 (77.8%)
Post-term (≥ 41 weeks)	0	1 (1.2%)	1 (0.6%)
Delivery Mode			
Cesarean section	34 (42.0%)	31 (38.2%)	65 (40.1%)
Vaginal delivery	47 (58.0%)	50 (61.8%)	97 (59.9%)
Birth Assistant			
Obstetrician	42 (51.8%)	45 (55.6%)	87 (53.7%)
Midwife	37 (45.7%)	35 (43.2%)	72 (44.4%)
Nurse	0	0	0
Physician	2 (2.5%)	1 (1.2%)	3 (1.9%)
Traditional birth attendant	0	0	0
Birth Weight			
≤ 2,500 gr	23 (28.4%)	25 (30.9%)	48 (29.2%)
> 2,500 gr	58 (71.7%)	56 (69.1%)	114 (70.8%)

IDR, Indonesian Rupiah.

The clinical-based diagnosis was made in most cases (90.1%), while cytogenetic-based testing was only done for less than 10% of cases. The diagnosis was established in the very early postnatal period (0–3 days) in 41.4% of cases and in less than one month in 13% of cases. In infancy (1–12 months), the majority of diagnoses was made within 1–5 months (26.5%) and 13% were made within 6–12 months. The fewest diagnoses (6.2%) were established at the age of 12 months and more (Figure 1B).

Table 2. Physical and clinical characteristics of participants

Characteristics	Boy (*n*, %)	Girl (*n*, %)
Musculoskeletal Problems		
*Hypotonia**	72 (88.9%)	68 (84.0%)
No hypotonia	9 (11.1%)	13 (16.0%)
Head and Neck		
*Epicanthal folds**	78 (96.3%)	78 (96.3%)
*Upslanted palpebral fissures**	77 (95.1%)	77 (95.1%)
*Protruding tongue**	52 (64.2%)	61 (75.3%)
Microcephaly/flat occiput	56 (69.1%)	51 (63.0%)
Flat nasal bridge	75 (92.6%)	77 (95.1%)
Short neck	57 (70.4%)	54 (66.7%)
Hand		
*Simian/single palmar crease**	63 (77.8%)	63 (77.8%)
Clinodactyly	70 (86.4%)	68 (84.0%)
Short of 5th finger	45 (55.6%)	37 (45.7%)
Feet		
*Sandal gap**	74 (91.4%)	72 (88.9%)
Flat foot	45 (55.6%)	46 (56.8%)
Syndactyly	2 (2.5%)	5 (6.2%)

*The main physical features (Devlin and Morrison, 2004).

3. Discussion

The survival rate is critical in children born with Down syndrome, especially in the first year of life, where the morbidity rate is very high (*13*). Sociodemographic characteristics and healthcare accessibility can affect survival rate. Previous report highlights newborn characteristics, including gestational age, birth weight, and comorbidities, because they can predict the survival rate. Individuals with Down syndrome have three times the mortality rate compared to typical individuals, and gestational age is one of the predictors of survival in Down syndrome (*9*). This study showed that most children were born full-term, and only 21% were born pre-term. Normal birth weight is a paramount concern for a baby with Down syndrome. The survival rate is low in Down syndrome for those who are born weighing less than 2,500 grams compared to those with normal birth

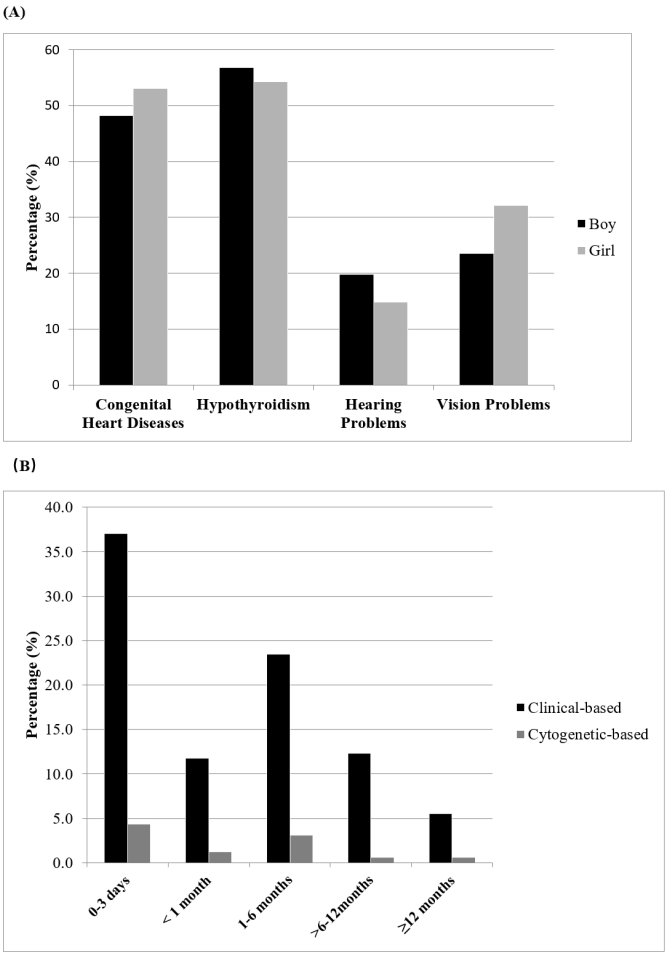

Figure 1. (A) The frequency of associated-medical conditions; (B) Age at diagnosis and frequency of diagnosis-based assessment.

weight. In addition, morbidity was increased in babies with very low birth weight (*9*). Our study counted low birth weight in approximately 30% of cases.

Congenital heart disease is found in about half of the children. A systematic review demonstrated that the prevalence of congenital heart disease in children with Down syndrome is estimated at around 66.1% (*14*). Previously, the mortality rate among Down syndrome with congenital heart disease was 14.1% (*15*). In addition, Down syndrome children have a higher risk of having thyroid disorders; therefore, the American Academic of Pediatrics (AAP) recommends thyroid screening at birth, at six months, and annually starting from one year (*16*). In our study, we found that more than half of the participants had thyroid problems. However, newborn screening is still not widely available in Indonesia; thus, not all children with Down syndrome undergo basic screening, and only some have thyroid examinations. Hearing problems affected almost 20% of cases. This result corresponds with a previous study that found in 22%-30% of cases having transient hearing loss (*17*), there was a double impact on speech and language development, resulting in low cognitive performance and mental age growth and outcome (*18*). In our study, vision problems were more prevalent in girls (32%) than boys (23.5%).

This study found the prevalence of hypotonia, epicanthic folds, up-slanted palpebral fissures, and "sandal gap" to be very high. In addition to Devlin and Morrison's clinical criteria, flat nasal bridge and fifth finger clinodactyly were found in high frequency. This may be due to the variability of clinical characteristics and also the type of Down syndrome karyotype, in which the mosaic cases show a broader spectrum of clinical characteristics. Unfortunately, only 10% of cases in this study were diagnosed based on chromosomal analysis.

In Indonesia, prenatal diagnosis has not been routinely offered in public healthcare facilities (primary or referral) because the national health insurance does not cover prenatal screening and other prevention programs. Moreover, there are still interregional disparities in primary antenatal care in Indonesia (*19*). Consequently, only some individuals who have a risk of having major congenital anomalies can afford to pay out of pocket to do prenatal testing/diagnosis. In our study, advanced maternal age was found in more than half of cases, and none of those completed the prenatal screening during pregnancy. Lacking awareness of healthcare professionals combined with limited health insurance coverage may decrease the opportunity to offer prenatal screening in advanced maternal age, even though international guidelines of prenatal testing recommend performing the aneuploidy screening test for advanced maternal age (*20*).

Regarding the birth assistant, obstetricians were the most prevalent birth assistants (53.7%), followed by midwives at 44.4% of cases in this study. Early postnatal diagnosis of Down syndrome is critical to prompt and early intervention. Our study documented that 41.4% of cases were clinically diagnosed at birth to 3 days after birth. Unfortunately, 6.2% of cases were diagnosed after 12 months of age. The characteristics of Down syndrome are less specific in newborns than in children. Hypotonia is the most striking characteristic, along with the manifestation of feeding problems and failure to thrive in newborns. Besides, small ears, "sandal gap", and nuchal skin fold are the most reliable and discriminative signs (*21*). Delays in diagnosis leads to decreases early identification and management of co-morbidities, which may worsen the clinical conditions.

This study has some limitations. This study was conducted on children with Down syndrome who were admitted to a pediatric clinic due to their critical co-morbidity. Thus, cases from primary care or in the community may not have been accounted for, and the critical problem may be bigger than shown in this study.

In conclusion, this study highlights some crucial issues in terms of Down syndrome in a developing country where the genetic laboratory is not accessible for the majority of the population, leading to delayed diagnosis in almost half of the cases and in increase in the frequency of co-morbidities. Advanced maternal age accounts for the majority of cases, and lack of cytogenetic laboratory assessment to confirm the diagnosis. Furthermore, based on a previous report that the life expectancy for Down syndrome is nearly 60 years, it is worth improving their quality of life by enhancing functional cognitive and adaptive outcomes and preventing their decline by identifying and managing the factors that may contribute to it, such as the co-morbidities.

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The epidemiology and healthcare burden of rare diseases requiring hospitalisation among adult patients in Langkawi, Malaysia: Insights from a pilot study

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SUMMARY In Malaysia, rare diseases affect fewer than 1 in 4,000 people. As of 2020, 491 rare diseases have been recorded in Malaysia, but with limited epidemiological data. As the first study in Malaysia, this retrospective cohort study examined the epidemiology and admission-related healthcare costs for adult rare disease patients in Langkawi. Among the 38 patients, rheumatological rare diseases topped the list (39.5%). The annual admission rate for rare diseases was 0.9%. Langkawi patients had lengthy hospital stays (9.7 days) and a 7.9% mortality rate. 23.7% of patients defaulted to follow-up, and 7.9% were referred to a tertiary hospital due to inadequate equipment or speciality care. Admission costs were Malaysian Ringgits (MYR) 244,598.63 (~US Dollars (USD) 51,280), with 80.2% from medication. The average healthcare resource utilisation was MYR 6,436.81/patient/year (~USD 1,350/patient/year).

Keywords rare disease, hospitalisations, healthcare costs, epidemiology, access to treatment

1. Introduction

Rare diseases in Malaysia are defined as conditions affecting fewer than 1 in 4000 people. As of August 2020, the Malaysian Rare Disease List has recorded 491 rare diseases (*1*). However, comprehensive data on their geographical and ethnic distributions within the country is lacking.

Rare diseases come with a substantial economic burden, with costs per patient per year (PPPY) being approximately ten times higher than mass-market diseases (US Dollars (USD) 266,000 PPPY versus USD 26,000 PPPY) (*2*). Orphan drugs are medicinal products designed to treat, prevent or diagnose rare diseases. Access to orphan drugs is limited, especially in Malaysia, where treatments like enzyme replacement therapy can cost more than Malaysian Ringgits (MYR) 700,000 (~USD 152,500) annually (*3*). However, funding constraints, particularly in district hospitals such as Hospital Sultanah Maliha in Langkawi, make it challenging to provide orphan drugs.

Geographical barriers further complicate access to treatment for rare diseases, especially in Langkawi, an island only accessible by air or sea. There is only one hospital in Langkawi, limiting treatment options for rare disease patients. The island's economy mainly relies on tourism, making it difficult for patients to allocate extra funds for healthcare-related travel outside Langkawi (*4*).

This retrospective cohort study aimed to describe the epidemiology and admission-related healthcare costs of managing adult patients with rare diseases in Langkawi from the perspective of the Ministry of Health (MOH) Malaysia, given that Hospital Sultanah Maliha is a public hospital under MOH Malaysia.

The patients recruited in this study from September 2021 to March 2023 were 13 years old and above, including those newly diagnosed with rare diseases during their current admission but not yet receiving treatment and those previously diagnosed and on treatment. Patients admitted for reasons unrelated to their rare diseases (*e.g.*, elective admission for arteriovenous fistula creation) were excluded.

The cost evaluation conducted in this study pertains to the year 2023. A micro-costing approach was employed, allowing for the quantification of each cost component associated with the treatment. From the provider's perspective, treatment costs were gathered using a 'bottom-up' approach. All costs

were denominated in MYR as of 2023. The average hospitalisation cost per patient was calculated by dividing the total hospitalisation costs of all patients by total number of patients. Detailed information about the items in each cost category can be found in the Supplemental Table S1 (*http://www.irdrjournal.com/action/getSupplementalData.php?ID=200*).

This study has been granted ethical approval by MREC MOH Malaysia (NMRR ID-23-00353-BDS).

2. Access to treatment

Rheumatological rare diseases top the rare disease list (39.5%), mostly suffering from systemic lupus erythematosus (SLE) (n = 11). The sociodemographic data and types of rare diseases of the patients are in Table 1. Among the 38 patients studied, 18 (47.4%) were newly diagnosed with a rare disease upon admission, while the others had a median of 1.5 years post-diagnosis. The annual admission rate for rare diseases in Langkawi was 0.9% (61 out of 6,773 admissions), with a higher rate among females at 1.7% (58 out of 3,369 admissions). Moreover, 13 (34.2%) patients experienced readmissions, a figure comparable to the 32% readmission rate in the USA (*5*). The readmission rate can be as many as five admissions within a year. Langkawi patients had longer hospital stays (9.7 days) compared to the other countries (6.1 days in Hong Kong and 6.3 days in the USA) (*5,6*), and it was double the duration among patients who required antimicrobial therapy (24 versus. 12 days, p = 0.001).

On the other hand, 9 (23.7%) patients had defaulted follow-up. These defaulted patients were significantly younger, with an average age of 31 compared to 44 (95% CI: 1.15 to 23.91; p = 0.033). Meanwhile, the mortality rate is double that reported in the literature (n = 3, 7.9% versus 3.9% in the USA) (*5*). 7.9% of patients were referred to a tertiary hospital due to inadequate equipment or speciality care. The rest of the patients (84.2%) were discharged home. A majority of the patients (n = 31, 81.6%) were receiving immunosuppressant treatment for their rare diseases, with corticosteroids (n = 25, 65.8%) being the most prescribed. With the high percentage of immunosuppression, 23 (60.5%) patients had to be treated with antimicrobials for infections. The other treatment modalities received by the patients include blood product transfusion, oxygen supplementation, and haemodialysis.

Table 1. Sociodemographic data and type of rare diseases of the study participants

Variables	*n* (%)
Sex	
Male	3 (7.9)
Female	35 (92.1)
Ethnic	
Malay	36 (94.7)
Chinese	2 (5.3)
Age group (years)	
13 - 29	10 (26.3)
30 - 49	17 (44.8)
50 - 64	7 (18.4)
≥ 65	4 (10.5)
Marital status	
Unmarried	10 (26.3)
Married with no children	4 (10.5)
Married with children	23 (60.6)
Divorced / Widowed	1 (2.6)
Employment status	
Employed	14 (36.8)
Unemployed	14 (36.8)
Not in labour force	10 (26.3)
Place of stay (distance from hospital)	
Kuah (6 km)	13 (34.1)
Ulu Melaka (10 km)	8 (21.1)
Padang Matsirat (11 km)	4 (10.5)
Kedawang (13 km)	8 (21.1)
Bohor (13 km)	2 (5.3)
Ayer Hangat (20 km)	3 (7.9)
Type of rare diseases	
Rheumatological (MCTD, rheumatoid meningitis, scleroderma, SLE)	15 (39.5)
Haematological (AIHA, Evans syndrome, HbH disease, PNH)	10 (26.3)
Neuromuscular (GBS, NMOSD, NORES)	7 (18.4)
Endocrine (primary adrenal insufficiency)	4 (10.5)
Dermatological (pemphigous vulgaris)	1 (2.6)
Renal (minimal change disease)	1 (2.6)

AIHA: autoimmune haemolytic anaemia; GBS: Guillain-Barré syndrome; MCTD: mixed connective tissue disorder; NMOSD: neuromyelitis optica spectrum disorder; NORES: new-onset refractory status epilepticus; PNH: paroxysmal nocturnal haemoglobinuria; SLE: systemic lupus erythematosus.

Table 2. Estimated costs (MYR) of rare disease management in Langkawi

Type of costs	Costs in MYR (USD)	Percentage of total costs (%)
Medication	196,161.63 (41,124)	80.20
Hospitalisation	29,600.00 (6,205)	12.10
Laboratory tests	12,891.00 (2,703)	5.27
Imaging and radiology	2,791.00 (585)	1.14
Blood products	2,700.00 (566)	1.10
Surgery and procedure	455.00 (95)	0.19
Total costs	244,598.63 (51,280)	100.00

3. Costs

The total admission costs of the recruited rare disease patients were MYR 244,598.63 (~USD 51,280), of which 80.2% were medication costs (Table 2). The total medication costs in this study (MYR 196,161.63 or ~USD 41,124) constituted 11% of the total medication costs for adult inpatients (all diseases) in the hospital during the study period (MYR 1,783,013.57 or ~USD 373,798). The treatments received by the patients include immunosuppressants, antimicrobials, fluid management, and other medications, in which immunosuppressants were the most expensive category of medication costs. On average, a rare disease patient in Langkawi pays

MYR 333.20 (~USD 70) per hospital admission, which only represents 5.2% of the annual hospitalisation cost per patient related to rare diseases borne by the hospital (MYR 6,436.81 or ~USD 1,350).

4. Discussion

Our data revealed almost 50% of new diagnoses (18 out of 38 rare disease patients admitted) related to rare diseases per year in Langkawi. This exceptional finding prompted us to investigate and report this matter, as many cases may still be undiagnosed and warrant a more systematic screening system. Those geographically isolated patients might have limited understanding of their conditions and lack medicinal support, leading them to opt for traditional or alternative medicines without a proper diagnosis in the hospital.

To our knowledge, this study represents the first cost analysis of managing rare diseases in Malaysia after a comprehensive literature search using PubMed Central, PubMed/Medline, Cochrane Review, Springer Link, and Lippincott Williams & Wilkins journals. Our findings showed that the total economic burden of hospitalised patients with rare diseases at Hospital Sultanah Maliha approaches MYR 245,000 (~USD 53,000) over one and a half years, signifying a considerable strain on the hospital's resources. A financial study in Langkawi showed that 60.9% of the population earns a monthly income of MYR 3,500 (~USD 760) or less (*7*). This income instability could prevent patients from seeking further healthcare treatment outside Langkawi.

Following the founding of the National Framework for Rare Disease, the Malaysian MOH adopted the Patient Access Scheme (PASc) to broaden patient access to high-cost medicines (*8*). PASc is a collaborative initiative between pharmaceutical companies and the MOH Malaysia. This programme incorporates innovative pricing agreements to improve cost-effectiveness and ensure broader patient access to specific medications. Despite PASc use to procure orphan drugs, its potential impact is restricted to a small number of patients.

Most previous pharmacoeconomic studies on rare diseases evaluate all cost categories, including direct costs, indirect costs and mortality costs. However, our study focused exclusively on direct medical costs. While the current public healthcare system is still unable to cope with the rising demands of rare disease treatment costs, specific groups of patients, for example, those with more comorbidities, need to be prioritised via a systematic process of orphan drug supply.

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The history of the Japanese Society for Neuro-infectious Diseases: Foundation, objectives, and legacy

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SUMMARY The Japanese Research Group for Neuro-infectious Diseases was founded in August 1996, and by 2004 it had evolved into the Japanese Society for Neuro-infectious Diseases. The Society focuses on neuroinfectious conditions (*e.g*., encephalitis/encephalopathy, myelitis, and meningitis), providing a venue for academic presentations and exchanges. Clinical guidelines for major neurological infectious diseases are also published by the Society, in order to meet the social demands of each era. Although the threat of herpes simplex encephalitis has declined due to acyclovir's introduction, the frequency of encephalitis or peripheral neuropathy caused by varicella-zoster virus is increasing. In Japan, prion disease, human T-cell leukemia virus-1 (HTLV-1)-associated myelopathy (HAM), subacute sclerosing panencephalitis (SSPE), and progressive multifocal leukoencephalopathy (PML) are designated as intractable diseases. The incidence of prion disease is 1.8/1,000,000 individuals, with the sporadic type accounting for 80%. Prion disease is fatal, and effective medications are awaited. HAM's prevalence is ~3/100,000 individuals, with a male-to-female ratio of 1:2–3. HAM is common in western Japan, including Kyushu and Okinawa. The prevalence of PML is rising with the spread of both immunosuppressive therapy for transplantation and treatment for multiple sclerosis. From late 2019 through 2020, the world faced a global outbreak of coronavirus disease 2019 (COVID-19) due to virus mutations, and the threat of new mutations persists. Close attention should be paid to the emergence of new neurological infections that could arise from abnormal weather patterns and/or a decline in immune function due to aging.

Keywords herpes simplex encephalitis, influenza encephalopathy, Creutzfeldt-Jakob disease/prion disease, HTLV-1 associated myelopathy, clinical guideline

1. Introduction

Neuro-infectious diseases including acute and subacute forms of encephalitis, encephalopathy, myelitis, and meningitis are not common but they are intractable conditions in which delays in diagnosis and treatment can result in prolonged illness, severe complications, or death. Prion disease, human T-cell leukemia virus-1 (HTLV-1)-associated myelopathy (HAM), subacute sclerosing panencephalitis (SSPE), progressive multifocal leukoencephalopathy (PML), and Bickerstaff brainstem encephalitis are designated as intractable and rare diseases, *i.e*., *nanbyo* in Japanese.

Herpes simplex encephalitis (HSE) or influenza encephalopathy was a threat in the early 1990s (*1,2*), and disparities in its diagnosis and treatment initiation across facilities led to medical lawsuits (*3*). To address this problem, the first research meeting of the Japanese Research Group for Neuro-infectious Diseases was convened in 1996. At the eighth research meeting in 2003, the Research Group was revised as the Japanese Society for Neuro-infectious Diseases. The Research Group and the Society both issued clinical practice guidelines in response to the societal needs of each era and have provided useful resources for general practitioners.

The current incidence of HSE remains unchanged at 3/1,000,000 individuals (*4*), but the threat posed by HSE declined with the introduction of acyclovir. Conversely, the incidences of the HSE-related diseases anti-N-methyl-D-aspartate (NMDA) encephalitis and paraneoplastic encephalopathy are increasing. The incidence of prion disease, a fatal disorder, is 1.8/1,000,000 individuals, with 80% being sporadic cases; the development of an effective treatment for prion disease is awaited (*5*). The lifetime incidence of HAM among HTLV-1 carriers is 0.3%, and adult T-cell leukemia/lymphoma (ATL) is diagnosed when abnormal lymphocytes exceed 5% in

white blood cells of peripheral blood (*6*). The incidence of PML is increasing with the widespread use of immunosuppressive therapy for transplantation and with the treatment of multiple sclerosis (MS) (*7*).

From late 2019 to 2020, the global outbreak of coronavirus disease 2019 (COVID-19) (*8*), primarily a respiratory infection, led to the postponement of the Society's 25th Congress to 2021, and the Congress was successfully held up to its 27th edition in October 2023. We reflect on the approximately 30-year journey of the Japanese Research Group and Society for Neuro-Infectious Diseases.

2. Overview of the Japanese Neuro-infectious Disease Research Group and Society

Table 1 summarizes the history of the Research Group and Society from the first issue of the Research Group's journal to the Society's journal, up to "Neuroinfection No. 23". We review the work of the Group and Society by dividing the history from 1996 to 2023 into three periods: *i*) the Japanese Neuro-infectious Diseases Research Group, 1996–2002; *ii*) the Japanese Society for Neurological Infectious Diseases, 2003–2012; and *iii*) the Japanese Neurological Infectious Diseases Society, 2013–2023, focusing on the president's lectures, special lectures, and clinical guidelines. This review was conducted in accord with the principles of the Declaration of Helsinki and was approved by our Hospital's Ethics Committee (Ron 23-104).

The titles of the Chairman's Lectures and Special Lectures from the 1st Research Group meeting to the 27th Society meeting reveal 10 titles concerning prion disease, six about viral encephalitis pathology, four for viral encephalitis, four for influenza encephalopathy, three regarding HAM, three concerning AIDS encephalopathy, single lectures about human herpesvirus-6 (HHV-6), SSPE, and PML, respectively; and others.

3. 1996–2002: The Japanese Neuro-infectious Disease Research Group

In February 1996, the first research meeting was hosted by Professor Toshiaki Takasu in Tokyo (*9*). It featured 94 members, approximately 160 participants, 34 general presentations, and two special lectures on "Influenza Encephalitis" and "Infectious Pathology of AIDS Encephalopathy". In 1999, the 4th Research Meeting took place under the leadership of Prof. Yasuto Itoyama in collaboration with Prof. Tetsushi Kitamoto of the Japan Neuro-virus Research Group. Notably, the two research groups merged in Sendai, the city credited with the discovery of the Sendai virus. The highlight of the 7th conference, orchestrated by Prof. Makoto Iwata in October 2002, was a significant international symposium titled "Emerging and Re-Emerging Infectious Diseases of the Nervous System". The conference also featured lectures on "Japanese Encephalitis" by Dr. S. Pradhan from India (*10*), "Nipah Virus Encephalitis" by Prof. Chong Tin Tan from Malaysia (*11*), and lectures on dengue fever and Hansen's disease.

4. 2003–2012: The Japanese Neuro-infectious Diseases Society

In 2003, our Research Group evolved into an Academic Society during its 8th meeting held in October in Ube City, under the leadership of Prof. Susumu Furukawa. The society boasted 300 members, approximately 200 participants, and 68 general presentations. Since 1996, there had been discrepancies among facilities regarding the diagnosis and treatment of HSE, leading to frequent medical lawsuits due to severe complications from delayed diagnoses (*3*), and there was thus an urgent need to establish medical guidelines. At the 9th conference in 2004, a workshop was organized to develop clinical practice guidelines for HSE, resulting in the publication of the HSE guidelines in an academic journal. This was followed by the release of a book that highlighted the importance of early acyclovir administration in suspected cases (*12*).

In 2007, the guideline for bacterial meningitis was published, overseen by Prof. Itoyama, and PDFs of guidelines for neurological infectious diseases that posed societal challenges such as influenza encephalopathy, prion disease, SSPE, and PML were released. These evidence-based clinical practice guidelines were developed in collaboration with The Health Labour and Welfare Science Committee, the Japanese Society of Neurology, and the Japanese Society of Pediatrics. These guidelines have been made available in PDF format, with

Table 1. Overview of the history of the Japanese Society of Neuro-infectious Diseases

1996	1st Japanese Neuro-infectious Diseases Group, Tokyo
1999	Co-sponsored by the 4th Research Group and Neuro-virus Research, Sendai
2003	8th Japanese Neuro-infectious Diseases Society, Ube City; 300 members, 200 participants, 68 general presentations
2014	19th Annual Meeting, co-hosted by the Japanese Neuro-immunology Society, Kanazawa City
2015	Herpes Simplex Encephalitis Clinical Guideline, Nankodo Co., 2017
2019	HTLV-1 Associated Myelopathy Clinical Guideline, Nankodo Co., 2019
2019	COVID-19 outbreak, pandemic
2020	Prion Disease Guideline (PDF) released
2021	Suspended due to the coronavirus pandemic
2022	27th Japanese Neuro-infectious Diseases Society, Yokohama City; 541 members, 393 registered participants, 48 general presentations

revised editions incorporating new findings published every three years.

5. 2013-2023: The Japanese Society for Neuroinfectious Diseases

The 18th Meeting, chaired by Prof. Hiroyuki Nunoi, featured a keynote lecture on the "Science of Virus and Host Reactions in Influenza Encephalopathy". The clinical practice guidelines developed during this period emphasized the importance of supportive care to maintain patients' general condition, with the aim of alleviating hypercytokinemia and recommending the administration of steroid hormone for cytokine storms. At the 22nd conference, Prof. Satoshi Kamei, chair of the organizing committee, discussed the "HSE Clinical Practice Guideline 2017" (*13,14*), which stipulated that acyclovir treatment should be started within 6 hours of symptom onset. The Guideline also mentioned the adjunctive use of corticosteroids to suppress inflammatory cytokines, in a short-term combination with antiviral drugs. Prof. Kamei also added the clinical features of a related disease NMDA limbic encephalitis associated with ovarian tumor, and Dr. Makoto Hara explained the pathophysiology of new nerve-cell surface antibodies in various types of immune encephalitis (*15*).

At the 23rd meeting in 2018, a special lecture was given by Prof. Hidehiro Mizusawa on the "Current Status and Prospects of Prion Diseases" (*5*). The frequency of sporadic prion disease in Japan at that time was 1.8/1,000,000 individuals per year. Prion diseases can be divided into familial, iatrogenic, and sporadic cases, with sporadic cases accounting for 80%. The special lecture highlighted two points: *i*) the conversion mechanism from normal prion protein to abnormal prion protein remains unknown, and *ii*) understanding the aggregation mechanism could offer insights into neurodegeneration. There is no specific treatment for prion diseases, and the development of such treatments is considered urgent.

The COVID-19 pandemic that began in December 2019 led to the postponement of the Society's 2020 conference. Although pneumonia was the primary concern in the early days of the pandemic, various neuromuscular complications including cerebrovascular disorders, meningoencephalitis, and myositis associated with COVID-19 have been reported (*16*).

The 25th meeting held in October 2021 and organized by Prof. Tetsushi Yoshikawa highlighted the practical use of real-time polymerase chain reaction (PCR) for the primary screening of several herpes genus species. In the 2024 educational lecture, Prof. Yoshikawa noted that the frequencies of encephalitis and encephalopathy due to HHV-6B reactivation during hematopoietic stem cell transplantation are 2%–3%, and the involvement of Epstein-Barr virus and cytomegalovirus in MS was described (*17*). Discussions held at the meeting revealed an increasing frequency of herpes zoster limbic encephalitis or varicella-zoster virus neuropathy (unpublished data, *18*). The 26th conference, led by Chairman Prof. Hiroshi Takashima, focused on how metagenomic analyses using next-generation sequencing could identify a new type of archaeal encephalitis (*19*), with symptoms improved by sulfamethoxazole and trimethoprim (ST) drug treatment.

The 27th meeting hosted in 2023 by Prof. Yoshihisa Yamano, saw the creation of a national HAM patient registry (HAM Net). The lifetime incidence of HAM among HTLV-1 carriers is 0.3%, and the prevalence of HAM is approx. 3 persons/100,000 people, with a male-to-female ratio of 1:2–3; HAM is common in western Japan, including Kyushu and Okinawa. Prof. Yamano's lecture entitled 'New Future of Neuroinfectious Diseases Revealed through HAM Research' delved into new pathophysiological insights (Figure 1) (*20*).

In conclusion, over its 30-year history, the Japanese Society for Neuro-infectious Diseases, through its journal *Neuroinfection* and academic conferences, has served as a platform for research and exchange on neuro-infectious diseases. Prion disease has been a focal point of attention, underscoring the urgent need for therapeutic drug development for this deadly disorder. With the impacts of abnormal weather and a super-aging population, further vigilance is warranted for various neurological infections. We hope that this legacy will

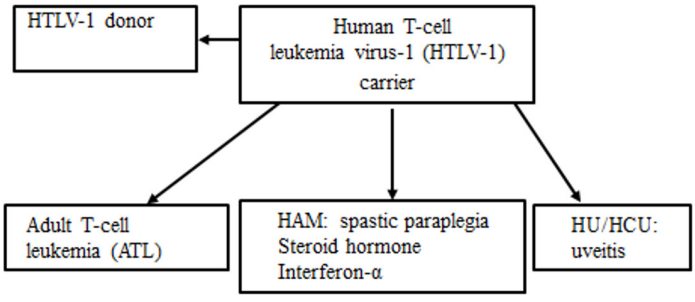

Figure 1. HAM and related disorders and current therapy. HAM patients exhibit signs of stiffness and spastic paraplegia in both lower limbs. The HTLV-1 antibody is positive in cerebrospinal fluid (CSF), and current treatments include steroids or interferon-alpha (IFN-α). Adult T-cell leukemia/lymphoma (ATL) is diagnosed when abnormal lymphocytes exceed 5% in white blood cells of peripheral blood. For HTLV-1-assciated uveitis (HU/HAU), steroids are effective. If an HTLV-1-positive individual is an organ transplant donor, testing for the HTLV-1 antibody is recommended.

continue for future generations as part of the history of the Japanese Society for Neuro-infectious Diseases.

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

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

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

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

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

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

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

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

3. Editorial Policies

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

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

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

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

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

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

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

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6. Manuscript Preparation

Manuscripts are suggested to be prepared in accordance with

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

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

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

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Examples are given below:

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

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

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

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

Example 4 (Sample web page reference):

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

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

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

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

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