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Rare disease publishing trends worldwide and in China: A CiteSpace-based bibliometric study

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SUMMARY: This study aimed to understand research trends, determine frontier topics, and explore the developments in and the differences between research conducted in China and the rest of the world. We analyzed the research status of rare diseases in China and globally over the past decade using bibliometric methods. We focused on rare disease literature indexed in the Web of Science (WoS) and China National Knowledge Infrastructure (CNKI) databases from January 2013 to December 2023. We selected studies based on inclusion and exclusion criteria. CiteSpace 6.1.R6 software were used to prepare knowledge graphs and perform comparative analyses of authors, institutions, content, and hot topics between both databases. A total of 10,754 articles from the WoS and 969 from the CNKI met the inclusion criteria. In the past 10 years, the diagnosis and treatment of rare diseases have been a common research focus in both China and the world. China has emphasized more on "orphan drugs". "Genes" and "management" were focused globally. The United States had the greatest number of publications. China ranks high in terms of publication volume and institutional ranking. Research interest in rare diseases has gradually increased worldwide, with European and American countries maintaining a leading position. China has made significant contributions. China's research is lagging compared to global trends, lacking collaboration with other countries. The diagnosis and treatment of rare diseases remain central themes, whereas genetic research, artificial intelligence, and sociological studies on rare disease populations are emerging as hot topics.

Keywords: rare diseases, orphan diseases, visualization, knowledge graph, CiteSpace

1. Introduction

Rare diseases refer to a group of clinically heterogeneous disorders characterized by considerably low prevalence and a small population of affected individuals (*1*). Different countries and regions have established different specific definitions for rare diseases. The U.S. Food and Drug Administration (FDA) defines rare diseases as "any disease or condition that affects < 200,000 persons in the United States (*2*)". The European Medicines Agency (EMA) defines rare diseases as "life-threatening or chronically debilitating conditions that affect no more than 5 in 10,000 people in the EU (*2*) ". According to The Report on the Definition of Rare Diseases in China 2021, China defines rare diseases as "diseases with a neonatal incidence rate of less than 1 in 10,000, a prevalence rate of less than 1 in 10,000, and a total affected population of less than 140,000 (*3*) ". According to estimates, rare diseases affect over 300 million patients worldwide, with over 7,000 rare diseases accounting for 10% of all human diseases. Among these, 80% are of genetic origin (*4*). However, currently, effective treatment methods are unavailable for more than 90% of rare diseases (*5*). The direct medical costs, non-medical costs, and productivity losses associated with rare diseases impose a significant burden on society (*6*). Therefore, the treatment and management of rare diseases have become important global public health issues (*7*).

According to statistics, the number of patients with rare diseases in China is estimated to be approximately 20 million, with an average of 200,000 new cases reported annually (*3*). Rare diseases are receiving increasing attention in China. In 2018, China released The First Batch of Rare Disease Catalog in China, which mentioned 121 rare diseases (*8*). This made China the first country to classify rare diseases using a specific list

(*9*). In 2023, China released The Second Batch of Rare Disease Catalog in China, which added 86 (*10*) new rare diseases to the list. Meanwhile, globally, developed countries such as the United States, France, and Germany have implemented national rare disease programs and orphan drug regulations and provide substantial funding to support research innovation and the establishment of healthcare systems for rare diseases (*11*). Academic research on rare diseases has experienced unprecedented and rapid development. WoS and CNKI databases are the most comprehensive databases for core journal indexing in China and worldwide. They can help identify scientific research trends from publications in different languages and country perspectives (*12*).

This study aims to provide a comprehensive overview of the research trends in rare diseases over the past decade using CiteSpace software. We summarize the development process in the field of rare diseases and provide directions for further advancements in this area.

2. Methods

2.1. Retrieval strategy

The data for this study were obtained from the WoS Core Collection and CNKI databases. For the WoS database, a topic-based search was conducted using the keywords "(rare disease) OR (rare cancer) OR (rare tumor)". The keyword "rare disease" was set as a "must inclusion", and the time range spanned from January 1, 2013, to December 31, 2023. The selected document types were "Review Article" and "Article."

For the CNKI database, the search strategy included the keywords "(rare disease (in Chinese)) OR (rare disorder (in Chinese)) OR (in Chinese))" for the topic search. The time range spanned from January 1, 2013, to December 31, 2023. The selected document type was "Journal Article".

2.2. Inclusion criteria

The inclusion criteria for the academic papers were as follows: the articles must have keywords such as "rare disease (in Chinese)", "rare disorder (in Chinese)", or "rare tumor (in Chinese)", and the research topic must be related to rare diseases or rare tumors. The inclusion was limited to academic journal papers available in the Chinese language for CNKI. No language restrictions were used for papers retrieved from the WoS.

2.3. Exclusion criteria

The exclusion criteria for literature selection were as follows: *i*) Duplicate publications, *ii*) Literature unrelated to the research topic of rare diseases or rare tumors, *iii*) Literature without relevant keywords and/or the complete text, *iv*) Conference papers, patents, newspapers, and project reports.

2.4. Literature screening and data extraction

Two researchers conducted a thorough review of the literature based on the predetermined inclusion and exclusion criteria. They screened the titles and abstracts to exclude irrelevant data. In case of discrepancies, a third person was consulted for consensus. The search results from CNKI were exported in "Refworks" format, whereas the results from WoS were exported using "Plain Text - Full Record and References" as the data source.

2.5. Analysis method

CiteSpace 6.1.R6 is a Java-based program that supports visual exploration and knowledge discovery in literature databases. The analysis was conducted using CiteSpace to aid the visualization of the research landscape (*13*). The time slice was set to 1 year, and the top N was set to 50. Pruning was set to Pathfinder and Pruning sliced networks. The analysis included the K-means clustering analysis of keywords and the identification of emerging research frontiers. The results were presented in the form of timeline graphs and keyword burst graphs in the visual interface of CiteSpace.

3. Results

3.1. Literature search results

A total of 11,347 papers were retrieved from WoS. After data inspection, deduplication, and cleaning, 10,754 papers were included in the study. These papers were exported in the form of "Plain Text - Full Record and References". An initial screening of CNKI yielded 2,152 papers. After further evaluation, 969 papers were selected

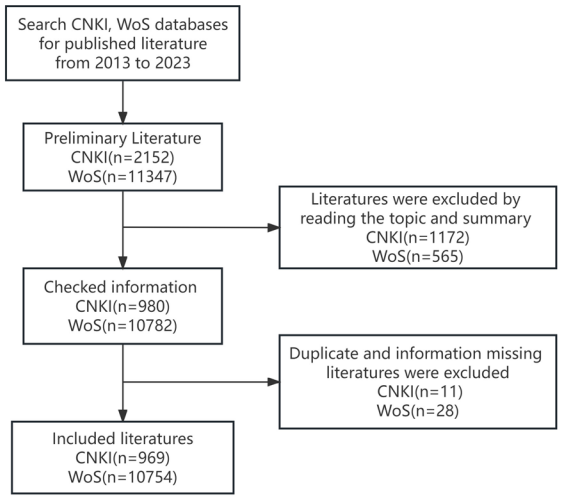

Figure 1. Flowchart of literature search and data incorporation.

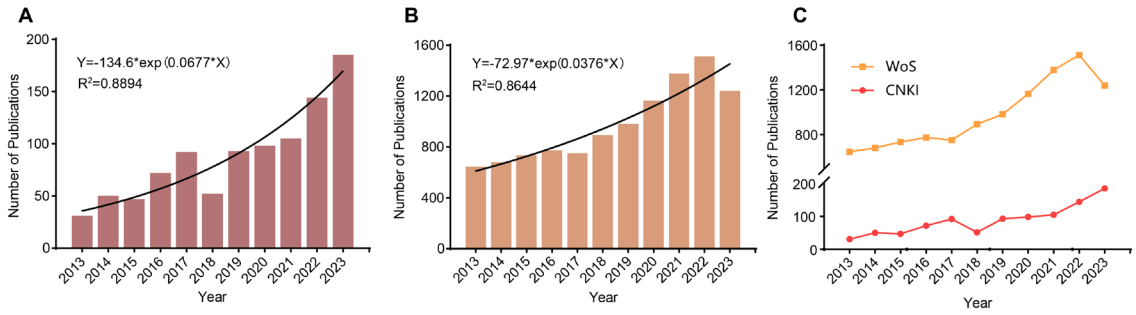

Figure 2. Chart of annual publishing and growth trends. (A) Trends in the number of publications per year in the CNKI database; **(B)** Trends in the number of publications per year in the WoS core database; **(C)** Comparison of publication growth trends between the CNKI and WoS core database.

and included in the study (Figure 1).

In the CNKI database (Figure 2A), the overall number of publications was relatively low and showed slow growth. There was a decline in 2018, followed by a recovery to normal levels. A rapid increase was observed from 2022. In 2023, the number of publications was 5.97 times greater than that in 2013. In the WoS database, the number of publications in the rare disease field showed a steady growth trend (Figure 2B). The growth rate increased significantly from 2018, although there was a marginal decline in 2023.

Based on a comparison of the domestic and international publication counts (Figure 2C), the field of research on rare diseases is continuously expanding both domestically and internationally. Compared to domestic journals, international journals show a higher level of attention and depth of research in this field.

Using a nonlinear index to fit the growth trend, the curve fitting equation for CNKI was set at $Y = -134.6*\exp(0.0567*X)$, $R^2 = 0.8894$; WoS is $Y = -72.97*\exp(0.0376*X)$, $R^2 = 0.8644$.

3.2. Database literature spatial distribution (core countries/institutions)

3.2.1. Countries and Regions

The country with the greatest number of publications in the WoS database was the United States, with 2,423 papers (22.5%). China ranked second with 1969 papers (18.3%), followed by Italy and Germany, both with over 1,000 papers (10.4% and 9.4%). However, there was a significant gap between the top two countries and the rest (Table 1). This indicates the core position of the United States in the field of rare disease research. China also demonstrated strong research capabilities. Among the top 10 countries, six were European countries. From the Network of Collaborating Countries (Figure 3), extensive and frequent collaboration was observed among European and American countries. However, currently, China has limited research collaborations with other countries. In terms of literature centrality,

Table 1. The top 10 productive countries with publications concerning rare diseases

Rank	Article counts	Centrality	Countries
1	2,423	0	USA
2	1,969	0	PEOPLES R CHINA
3	1,123	0	ITALY
4	1,020	0	GERMANY
5	881	0.01	FRANCE
6	821	0.07	ENGLAND
7	649	0	JAPAN
8	598	0.07	SPAIN
9	465	0.05	CANADA
10	379	0.09	NETHERLANDS

the Netherlands had the greatest intermediary centrality (0.09), indicating close collaborative connections with other countries. The Netherlands has not only published a large volume of research output but also achieved high-quality results in the field of rare disease research, thus playing a crucial role.

3.2.2. Research Institutions

Among publishing institutions (Table 2), Chinese research institutions have abundant research output in both domestic and international databases. The Beijing Union Medical College Hospital, Chinese Academy of Medical Sciences, as a top medical and research center in China, is the only institution that ranks among the top 10 institutions in both domestic and international databases. It houses the National Key Laboratory for Rare and Difficult Diseases and focuses on diseases such as Gitelman syndrome, transthyretin amyloidosis cardiomyopathy, and hereditary retinal degeneration. Zhejiang University has the greatest number of publications in the WoS database and primarily focuses on rare diseases such as spinal muscular atrophy and Wilson's disease. Combining the results of the analysis of countries in the previous section, it is evident that European and American countries hold critical positions in the field of rare disease research. Meanwhile, the research capabilities of China are noteworthy.

Figure 3. Network of collaborating countries. The circle indicates the country; the larger the size of the circle, the greater the number of publications from the country. Links between nodes describe cooperation between the countries.

Table 2. The top 10 productive institutions ranked by the numbers of publications

Rank	Article counts	Institutions (CNKI)	Article counts	Institutions (WoS)
1	74	Peking Union Medical College Hospital	151	Zhejiang Univ
2	61	China Medical University	132	Capital Med Univ
3	31	Peking University	127	Harvard Med Sch
4	31	Peking Union Medical College	126	Univ Milan
5	21	Shandong University	123	Mayo Clin
6	20	National Medical Products Administration	118	Univ Toronto
7	18	Shenyang Pharmaceutical University	105	Univ Penn
8	15	Shanghai Health and Health Development Research Center	98	Chinese Acad Med Sci
9	14	West China Hospital, Sichuan University	94	Sichuan Univ
10	12	Shanghai Jiao Tong University	90	INSERM

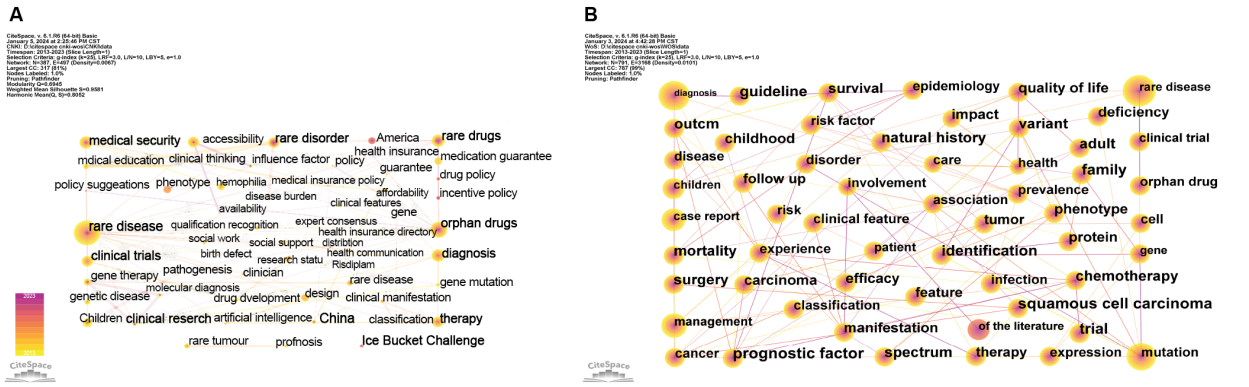

Figure 4. Network of primary keywords in publications. (A) Network of primary keywords in the CNKI database; **(B)** Network of primary keywords in the WoS core database. The circle indicates the keyword; the larger the size of the circle, the greater the frequency of the keyword.

3.3. Keywords

3.3.1. Co-occurrence of keywords

Keywords are a reflection of the core content of the literature. In this study, we selected co-occurrence network graphs of keywords with a frequency greater than 100 in the WoS database (Figure 4B). The top three keywords were "rare disease" (1,662), "diagnoses" (980), and "mutation" (833) (Table 3). "Management" (794) also had a high frequency. In the CNKI database (Figure 4A, Table 3), "rare disease" was also the most frequently appearing keyword (452), followed by "orphan drugs" (100) and "diagnosis" (38). A comparison of the two showed that "rare disease", "diagnosis", "therapy", and "Children" are among the

Table 3. The top 10 keywords ranked by frequency

Rank	Article counts	Centrality	Keywords of CNKI	Article counts	Centrality	Keywords of WoS
1	452	1.19	rare disease	1,662	0.01	rare disease
2	100	0.11	orphan drugs	980	0	diagnosis
3	38	0.05	diagnosis	833	0.01	mutation
4	35	0.02	therapy	794	0.01	management
5	31	0.01	medical security	619	0.01	disease
6	29	0.11	rare drugs	602	0.01	children
7	23	0.01	children	403	0.01	therapy
8	20	0.05	rare disorder	390	0	case report
9	19	0.01	clinical trial	380	0.01	expression
10	15	0.02	accessibility	377	0.01	cancer

top 10 keywords with the greatest frequency in both databases. This indicates that diagnosis, therapy, and children's rare diseases are common themes of concern in this field at both domestic and global levels, and they represent the research focus. However, among the high-frequency keywords in the domestic database, certain terms were related to orphan drugs and rare drugs, which were also treatment-related. In contrast, the international database focused more on gene-related directions such as "mutation" and "expression", as well as rare disease management. This reveals the different research perspectives between domestic and international studies in this field. Chinese research institutions may place a greater emphasis on drug development and application, whereas international research tends to explore the mechanisms underlying rare diseases and the social management of special populations.

3.3.2. Keyword clustering

Keyword clustering can reflect the different research focuses in a particular field. The smaller the clustering number, the more the keywords are included in that cluster. In the WoS database (Table 4), orphan drugs and whole-exome sequencing were the most prominent research directions. Case reports were the primary form of research output, indicating that with the improvement of medical standards and advancement of diagnostic methods, a greater number of rare disease cases are being reported. In the CNKI database (Table 4), orphan drugs remain a research focus. The comparison between the two databases reconfirmed the difference in research focus between domestic and international studies in the field of rare diseases. These research directions also indicate that international research on rare diseases has focused on findings at the molecular level, whereas Chinese journals continue to focus on treatment and medication, with less emphasis on the investigation of disease mechanisms. The development of the field of rare diseases in China is less advanced than the international community.

3.3.3. Keyword timeline graph

The timeline graph reflects the development of keywords within each cluster. In the WoS database (Figure 5B), the top ten keywords in terms of frequency have been appearing since 2013 and have maintained a high occurrence rate over the past 10 years. This indicates that the diagnosis, therapy, management, and genetic research of rare diseases constitute the foundation of this field. On this basis, medical genetics (2019), genomics (2018), and public health (2020) have advanced considerably, aiding rare disease research at a deeper investigative level. In the CNKI database (Figure 5A), high-frequency keywords such as "rare disease", "orphan drugs", and "rare drugs" related to medications have appeared since 2013 and have laid the foundation for subsequent research directions. Keywords such as "diagnosis", "therapy", and "clinical trials" only appeared first in 2015-2016, exhibiting a lag compared to international trends. Keywords related to rare disease management and social support in the field of social sciences also appeared relatively late, indicating that China still lacks sufficient social attention and policy support for rare disease populations. Based on observations of the keyword burst graph (Figure 6), the Ice Bucket Challenge, which went viral on social media for promoting awareness of amyotrophic lateral sclerosis (ALS), once created a wave of enthusiasm in China but disappeared within a year. This indicates that while marketing-style dissemination can increase social awareness of rare diseases, it does not significantly impact social security in rare disease populations. Currently, the research focus is concentrated on medical security, and governmental influence may significantly improve the lives of rare disease populations in China.

3.4. Authors

In the WoS database, Taruscio, Domenica; Boycott, Kym M; and Baynam, Gareth were the top three authors in terms of publication volume (Table 5). The three authors constituted the center in the collaboration network, and their collaboration has gained prominence in the past 5 years (Figure 7B). Before this period, the author collaboration network was not significant, with the majority of authors producing independent work.

Table 4. The information of clusters about keyword co-citation analysis

Database	Clusters	Label	Terms
WoS Database	0	rare disease	rare disease; target therapy; desmoplastic small round cell tumor; tyrosine kinase receptor; fetal growth restriction | squamous cell carcinoma; targeted therapy; penile cancer; systemic therapy; retroperitoneal sarcoma
	1	orphan drug	rare disease; orphan drug; spinal muscular atrophy; real-life outcome data; single-arm trial | rare diseases; comparative effectiveness; evidence generation; patient-oriented outcomes; evidence synthesis
	2	case report	case report; langerhans cell histiocytosis; igg4-related disease; central diabetes insipidus; bone marrow | magnetic resonance imaging; myeloid sarcoma; sacral spine; primary testicular lymphoma; testicular cancer
	3	whole-exome sequencing	rare disease; whole-exome sequencing; genic intolerance; health ethics; government regulation | mutation; variant; protein; tool; common disease
	4	aortic dilatation	rare disease; aortic dilatation; bicuspid aortic valve; aortic dissection; aortic coarctation | pulmonary hypertension; lung cancer; von recklinghausen; neurofibromatosis type; viral coinfection
	5	oxidative stress	rare disease; oxidative stress; lipid metabolism; mitochondrial dysfunction; brain iron accumulation | gene expression; growth; model; mutation; mice
CNKI database	0	rare disease	rare disease; orphan drugs; rare drugs; rare disorder; diagnosis
	1	orphan drugs	orphan drugs; medicine policy; comparative analysis; analysis; price negotiation
	2	rare drugs	rare drugs; America; pharmaceutical companies; research and development; indication expansion
	3	Pathology	Pathology; gene therapy; clinical studies; diagnosis; rare disease
	4	children	children; therapy; artificial intelligence; clinical manifestations; classification
	5	medical education	medical education; rare disorder; clinical thinking; computer technology; teaching quality
	6	medical security	medical security; Hemophilia; affordability; ethical principles; medical insurance policy
	7	accessibility	accessibility; influencing factors; availability; strategy research; evaluation index
	8	clinical trial	clinical trial; recruitment; drug development; rare lung tumors; priority review
	9	China	China; guarantee; national rare disease registration system; cooperation; Gauchers disease

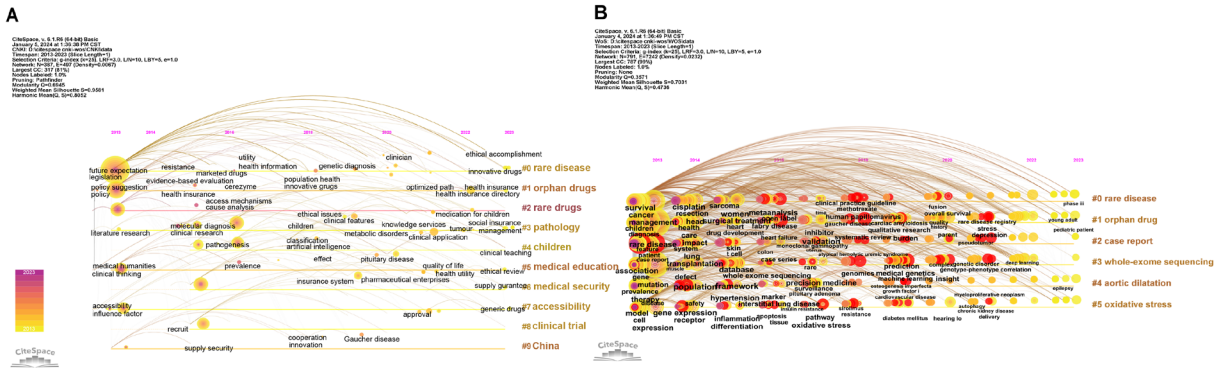

Figure 5. Timeline view of keywords. (A) Timeline view of keywords in the CNKI database. **(B)** Timeline view of keywords in the WoS core database. The circular nodes on the line represent the top three keywords with the greatest frequency of occurrence in this time slice. The timeline is shown at the top of the figure, and the year corresponding to the node is its publication time. The link between nodes represents the co-citation relationship.

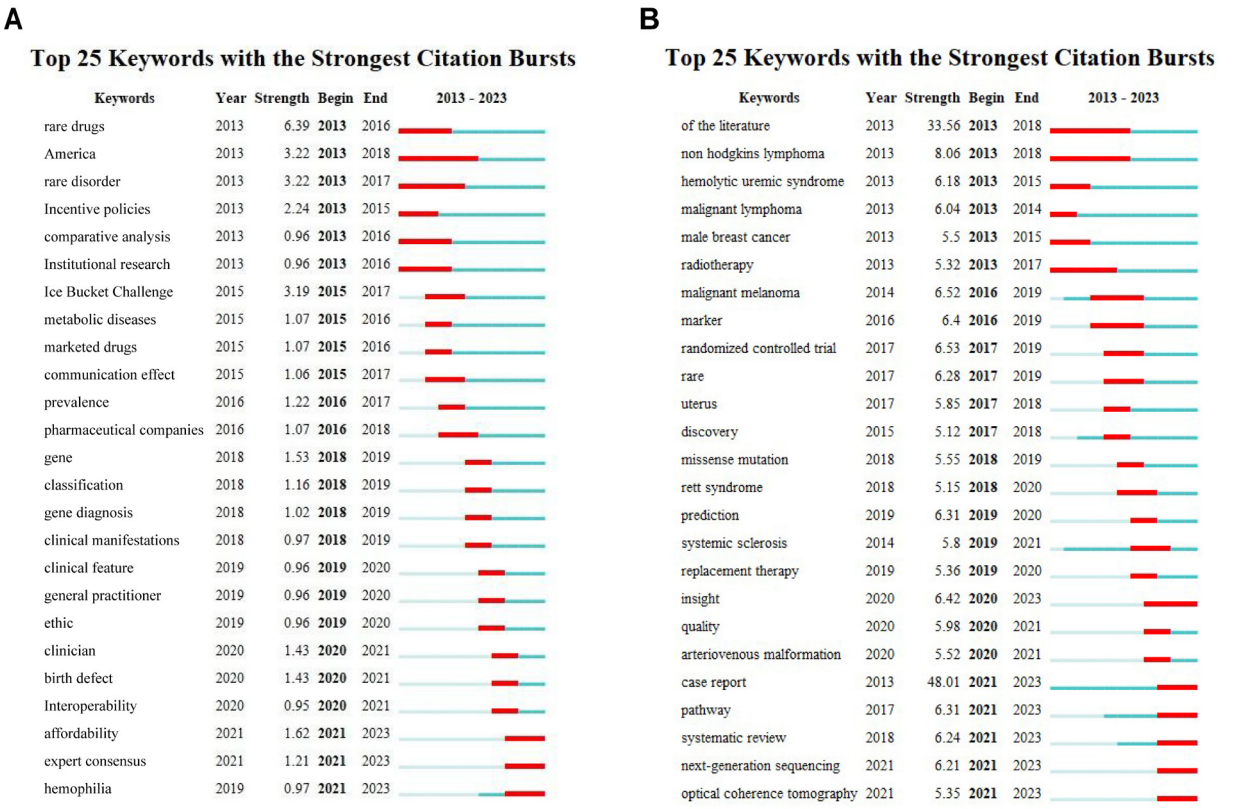

Figure 6. Twenty-five keywords with the strongest citation bursts. (A) The 25 keywords with the strongest citation bursts in CNKI; **(B)** The 25 keywords with the strongest citation bursts in the WoS core database. The blue line indicates the time axis, whereas the red segment on the blue time axis indicates burst detection, along with the start year, end year, and burst duration.

Table 5. The top 5 productive authors ranked by the numbers of publications

Article counts	Author	Rank	Article counts
26	Taruscio, Domenica	1	27
25	Boycott, Kym M	2	15
17	Baynam, Gareth	3	11
11	Robinson, Peter N	4	11
11	Lochmueller, Hanns	5	10
-	-	6	10

This indicates that contemporary research places greater emphasis on multi-team collaboration and multi-center studies. Taruscio, Domenica, Boycott, Kym M, and others have contributed to rare disease research for a long time, consistently producing research output, and they are important scholars in this field.

The author collaboration network reflected in the CNKI database is relatively close but shows clear stages (Figure 7A). In 2013-2014, Pei-Wen Wang and Jin-Ping Xie were the central authors in the network. The collaboration network centered around Shu-Yang Zhang, Bo Zhang, and Meng-Chun Gong lasted for a longer duration. The publication volume clearly indicated that this team has made outstanding contributions to the field of rare diseases (Table 5). However, their output decreased significantly in the past 3 years, and the formation of novel collaboration networks is not yet apparent.

3.5. Co-cited references

Highly cited references can indicate the hot topics in a research field. By analyzing these co-cited references, the dynamic changes in research topics within a specific time range can be identified. The top three co-cited references were "Estimating cumulative point prevalence of rare diseases: analysis of the Orphanet database (*14*)", "Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (*15*)", and "The mutational constraint spectrum quantified from variation in 141,456 humans (*16*)" (Table 6). The first-ranked reference was cited as many as 156 times. We used the Orphanet database to estimate the cumulative point prevalence of rare diseases. Of the 6,172 unique rare diseases, 71.9% were genetic, and 69.9% manifest in

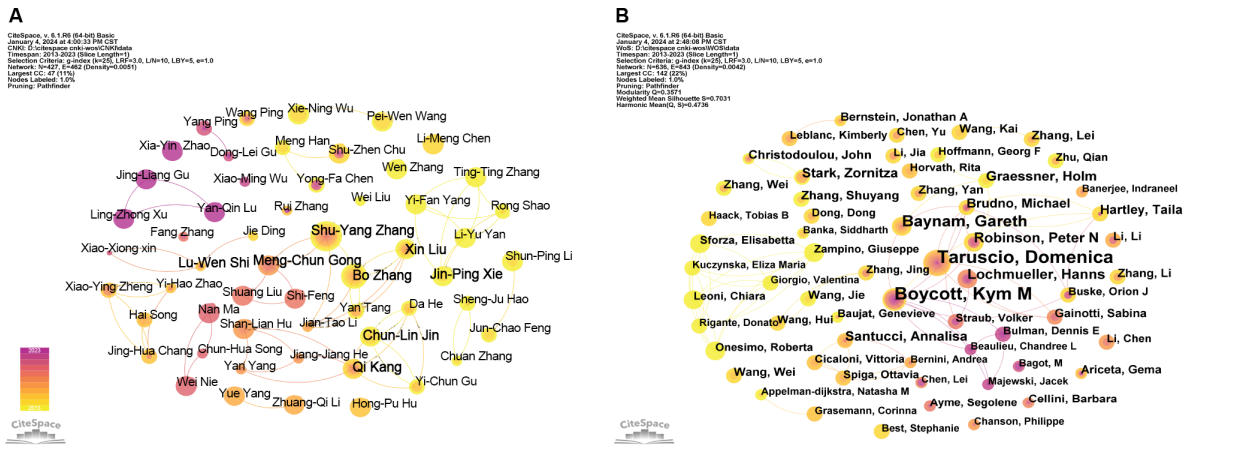

Figure 7. Co-author analysis. (A) Co-author network in the CNKI database; **(B)** Co-author network in the WoS core database. The circle indicates the author; the larger the size of the circle, the greater the number of publications from the author. The links between the nodes indicate cooperation between the authors.

Table 6. The top 10 cited references with the highest cited frequency

Freq	Burst	Centrality	Author	Source	DOI
170	28.18	0.02	Wakap SN	EUR J HUM GENET	10.1038/s41431-019-0508-0
110	34.05	0.01	Richards S	GENET MED	10.1038/gim.2015.30
86	13.74	0.01	Karczewski KJ	NATURE	10.1038/s41586-020-2308-7
83	20.45	0.01	Lek M	NATURE	10.1038/nature19057
60	3.62	0.02	Wright CF	NAT REV GENET	10.1038/nrg.2017.116
58	9.74	0.03	Ferreira CR	AM J MED GENET A	10.1002/ajmg.a.61124
57	0	0.02	Haendel M	NAT REV DRUG DISCOV	10.1038/d41573-019-00180-y
49	3	0.03	Boycott KM	AM J HUM GENET	10.1016/j.ajhg.2017.04.003
45	5.89	0.01	Landrum MJ	NUCLEIC ACIDS RES	10.1093/nar/gkx1153
43	17.32	0.02	Boycott KM	NAT REV GENET	10.1038/nrg3555
43	0	0.01	Rentzsch P	NUCLEIC ACIDS RES	10.1093/nar/gky1016

Table 7. The top 15 cited references with the strongest citation bursts

Freq	Burst	Centrality	Author	Source
110	34.05	0.01	Richards S	GENET MED
170	28.18	0.02	Wakap SN	EUR J HUM GENET
83	20.45	0.01	Lek M	NATURE
43	17.32	0.02	Boycott KM	NAT REV GENET
86	13.74	0.01	Karczewski KJ	NATURE
31	11.89	0.04	Kircher M	NAT GENET
26	10.43	0.01	Köhler S	NUCLEIC ACIDS RES
35	10.06	0.04	Richter T	VALUE HEALTH
23	9.91	0	Page MJ	PLOS MED
58	9.74	0.03	Ferreira CR	AM J MED GENET A
34	9.07	0.05	Köhler S	NUCLEIC ACIDS RES
34	9.06	0.02	Philippakis AA	HUM MUTAT
32	9.04	0.01	Zurynski Y	ORPHANET J RARE DIS
21	8.42	0.07	Girdea M	HUM MUTAT
21	8.42	0.02	Yang YP	NEW ENGL J MED

childhood. The second- and third-ranked references were both related to genomics.

Table 7 presents further analysis of hot topics and progress in the field of rare diseases over the past 10 years. This study selected the top 15 references with the strongest citation bursts. Among them, six articles were related to genomics (*15-20*), and three articles reported database-related content, including those related to the Orphanet (*14*) and HPO databases (*21, 22*). Two articles introduced disease-related platforms, namely The Matchmaker Exchange (*23*), a platform for rare disease gene discovery, and PhenoTips (*24*), a software for patient phenotype analysis for clinical and research purposes. The remaining articles cover topics

Table 8. The top 4 cited references with the highest centrality

Freq	Burst	Centrality	Author	Source	DOI
22	3.9	0.11	Benson MD	NEW ENGL J MED	10.1056/NEJMoa1716793
13	6.73	0.09	Bamshad MJ	NAT REV GENET	10.1038/nrg3031
8	0	0.09	Gurovich Y	NAT MED	10.1038/s41591-018-0279-0
21	8.42	0.07	Girdea M	HUM MUTAT	10.1002/humu.22347

such as writing standards for reviews (*25*), disease terms definition (*2*), social science research (*21*), and reviews (*26*) on rare diseases. Evidently, genomic research is a hot topic in the field of rare diseases. Contribution to disease diagnosis and treatment using big data and artificial intelligence is also a growing trend. With social progression, minority groups are gradually receiving more attention, leading to an increase in social science research on rare disease populations.

Centrality can indicate the importance and influence of literature in a specific field. In this study, the top four cited references with the greatest centrality were selected (Table 8). Among them, one article had a centrality ≥ 0.1. This article introduced the use of exome sequencing to identify disease-causing genes (*27*), reaffirming that genetics is a research hotspot and development trend in this field.

4. Discussion

4.1. Comparison of domestic and foreign databases

An analysis of the progressive knowledge graph in the field of rare diseases over the past 10 years shows that the field of rare disease research is entering a phase of rapid development. However, there are certain differences in the research data between domestic and foreign databases. In terms of the number of publications, the number of publications included in WoS over the past 10 years is ten times greater than that in CNKI. This could be explained by the fact that the publications were from global journals, whereas most journals in CNKI were from China. In terms of the growth rate, WoS has been growing rapidly since 2018, whereas CNKI showed a significant growth acceleration in 2022. This may be attributed to high-quality research from China likely being published internationally.

From the perspective of focus, the frequency ranking of keywords and clustering in CNKI indicate that domestic journals in China focus more on rare disease drugs, whereas foreign journals focus on genetics and therapies. China's policies may help explain this difference. European countries started using Managed Entry Agreements (MEAs) to manage rare disease drugs as early as 2013 or even before that. National authorities have the flexibility to adjust funding support, and pharmaceutical companies can ensure the normal market access of drugs considering cost-benefit conditions (*28*). In recent years, policies have further improved, and some countries have developed specific programs to evaluate the approval of rare disease drugs. For example, in Italy, the approval process for orphan drugs is stated to be completed within 100 days (*29*). In comparison, China's corresponding policies are less advanced. Following the announcement of the first batch of rare disease catalogs in 2018, in April 2019, *The Drug Administration Law of the People's Republic of China* was enacted to encourage development of innovative rare disease drugs and prioritize evaluation of approval (*30*). However, it did not specify a timeframe.

Policy orientation is closely related to academic development. Developed countries such as Europe and the United States have established comprehensive and mature systems for implementing research, approval, and market access of rare disease drugs. These countries are also the primary forces in research and production of rare disease drugs. Therefore, they conduct more genetic research, uncovering disease mechanisms and explore new signaling pathways and mechanisms of action for the development of new drugs and therapies. Conversely, in China, most rare disease drugs are imported, and issues related to drug accessibility and market access still require improvement, despite the introduction of multiple policies in recent years to encourage independent drug research and development. However, the process is lengthy, and significant achievements may only be reported years later.

4.2. Research hotspots in the field of rare diseases

4.2.1. Rise of social science research

Patients with rare diseases often bear a dual burden of physical and psychological challenges. In the era of the biological-psychological-social medicine model (*31*), the diagnosis and treatment of diseases are no longer limited to technical aspects; it is also important to focus on the psychological well-being and social identity of patients. This has led to the development of medical humanities research in the field of rare diseases. Among the top 15 highly cited references, Zurynski Y's cross-sectional study highlighted the reasons and consequences of delayed diagnosis in children with rare diseases. It emphasizes the need for healthcare professionals to provide psychological support to patients and for parents to prioritize genetic counseling and opt for rare disease-related education (*32*). Disease burden is also a prominent theme of research. In the

2019 US Rare Disease Economic Burden Assessment, excess expenditures were primarily attributed to hospital inpatient care, prescription drugs, and productivity losses in the labor market owing to absenteeism and early retirement (*33*).

In addition, the keyword "quality of life" appears over 200 times in the WoS database. A survey on the survival conditions of rare disease populations showed a positive correlation between informal social support and quality of life. Patients who received social assistance had better quality of life in the psychological and social domains than those who did not (*34*). "Management" and "healthcare coverage" are also frequent keywords. Countries worldwide are continuously updating their policies related to rare diseases (*35,36*). Processes to integrate rare disease populations into mainstream society are a global issue. In May 2021, the United Nations passed its first resolution on "Addressing the Challenges of Rare Disease Patients and Their Families," which covers various aspects, such as education, employment, poverty, gender inequality, and support for inclusion of patients with rare diseases in multiple societal dimensions (*37*). As social citizens, rare disease populations have the right to enjoy social welfare policies, equal access to education, and employment opportunities. This can help improve their community awareness and self-acceptance. At the societal level, it can alleviate socioeconomic and management burdens, indicating the progress of human civilization.

4.2.2. Application of artificial intelligence and big data

Owing to the small size of the rare disease population, misdiagnosis and mistreatment are common. Use of big data aids sharing and linkage of patient information worldwide. Using the powerful computational capabilities of artificial intelligence, the relationship between genes and disease onset can be determined, and potential drug targets can be explored. Therefore, establishing a rare disease information database is of great significance for diagnosis, treatment, and research. Orphanet is a widely used database with information on rare disease medical classifications, gene information, and epidemiological indicators, among other data. The most highly cited reference in this context uses accumulated data collected by Orphanet to estimate disease prevalence (*14*). The Matchmaker Exchange (MME), which mentioned in a high-burst cited reference, is a federated network connecting databases of genotypes and rare phenotypes using a common application programming interface (*23*). By 2022, which was 7 years since it was founded, the network had data from over 120,000 cases provided by more than 12,000 volunteers from 98 countries, with over 13,520 unique gene-to-gene matches established. New gene-disease connections are discovered every day (*38*). Over the past decade, artificial intelligence has been used in diagnosis and treatment of various diseases. For example, machine learning algorithms have been used to predict the clinical severity of progressive supranuclear palsy (PSP) by analyzing diffusion tensor imaging characteristics of the brain (*39*). Automatic assessment tools have been developed to predict speech disorders in patients with ALS based on speech acoustics and pronunciation samples (*40*). Big data and machine learning demonstrate the immense potential of artificial intelligence in the field of rare diseases. However, widespread implementation of these tools in clinical applications requires regulatory frameworks, evidence from clinical trials, and compliance with ethical guidelines (*41*).

Disease registration is also focused on both domestically and internationally. Owing to the challenges in collecting rare disease data, individualized tracking of each patient is typically performed using case registries. Collecting epidemiological data through registries helps monitor quality of management and patient prognosis processes while providing convenience for clinical research. Developed countries such as Europe and the United States have achieved a high level of refinement, regionalization, and networking in their registries. Examples include the European Rare Kidney Disease Registry (ERKReg) (*42*) and the European registry for patients with McArdle disease and other muscle glycogenoses (EUROMAC registry) (*43*) . In contrast, in China, rare disease registries have been developed relatively lately. The National Rare Disease Registry System (NRDRS), established in 2016, is expected to have a positive impact on epidemiological research within the country (*44*).

4.2.3. Genetic Research

Genomic research is one of the most important hotspots in the field of rare diseases. In keyword analysis, keyword clustering in CNKI includes the label of "gene therapy", whereas the high-frequency keywords in WoS include "mutation", "expression", and "gene", all of which appear more than 300 times. Among highly cited references in WoS, approximately half of the articles are related to genomics. The highest-ranked highly cited reference is a standard and guideline on interpretation of sequence variations. It uses various types of variant evidence, including population data, computational data, functional data, and segregation data, to classify variations into five standards: "pathogenic", "likely pathogenic", "uncertain significance", "likely benign," and "benign" (*15*). This article serves as a foundational declaration in the field of genomics. However, owing to the significant proportion of genetic diseases among rare diseases, development of this guideline also contributes to the standardized representation of gene mutations in rare disease genomics research, which is significant in determining gene-disease relationships.

Genomics is not only used to explore the etiology of rare diseases but also plays a role in development of therapeutic drugs and treatments as research progresses. Luxturna, a gene therapy drug using adeno-associated virus (AAV) vectors, is administered through subretinal injection to treat inherited retinal dystrophy caused by RPE65 protein deficiency. Patisiran is an oligonucleotide-based therapy for familial transthyretin amyloidosis (FTA) that inhibits misfolding of transthyretin protein, thereby impeding disease progression. Rare disease drugs based on gene therapies such as CAR-T and retroviral vectors have also been approved and launched in foreign markets (*45*). As more mechanisms are discovered, the scope of gene therapy will continue to expand, bringing new hope for treatment of rare diseases. However, adoption of gene therapy in China remains limited, and further research and development are needed before it can be widely used in clinical practice.

4.3. Key scholars and institutions in the field of rare diseases

In the CNKI database, the core author Shu-Yang Zhang stands out with a significantly high publication volume. As the President of Peking Union Medical College Hospital (PUMCH) and Vice President of Peking Union Medical College, the collaborative network centered around Shu-Yang Zhang has consistently produced output over the past decade. Research institutions associated with Shu-Yang Zhang, namely PUMCH and Peking Union Medical College, have also achieved remarkable results in the field of rare disease research in China. Shu-Yang Zhang is a leading scholar in the field of rare diseases in China, with most of his published articles focusing on rare cardiovascular diseases or rare disease policies in China. Examples include "Progress in the Diagnosis and Treatment of Rare Cardiovascular Diseases (*46*)" and "Construction and Application of China's National Rare Disease Registration System (*47*) ". The articles published by Shu-Yang Zhang are mostly systematic reviews and expert consensus articles. PUMCH, as the host institution of the National Key Laboratory for Severe and Rare Diseases, encompasses various disciplines such as oncology, surgery, obstetrics, and gynecology. Its achievements span multiple types of publications, including case reports, systematic reviews, and clinical studies.

In the WoS database, Taruscio D and Boycott KM are the authors with the greatest number of publications. The collaborative network centered around these authors is the only evident collaboration network in the WoS database. Taruscio D is affiliated with the Istituto Superiore di Sanita in Rome, Italy, whereas Boycott KM is affiliated with the Children's Hospital of Eastern Ontario in Canada. Both researchers have interests in genetics and experimental medicine. However, Taruscio D also focuses on public health management and healthcare services related to rare diseases, whereas Boycott KM has a stronger emphasis on genomics.

In the analysis of co-cited literature, Boycott KM has two highly cited and highly prominent articles: "International Cooperation to Enable the Diagnosis of All Rare Genetic Diseases (*48*)" and "Rare-disease genetics in the era of next-generation sequencing: discovery to translation (*18*)" . However, Taruscio D does not have any highly cited articles. This indicates that Boycott KM is a key scholar in the field of international rare disease research and an important member of the International Rare Diseases Research Consortium (IRDiRC).

From an institutional perspective, Chinese research institutions have demonstrated significant achievements in scientific research globally. However, collaboration with international institutions is yet to be established. Domestic and international institutions and scholars should strengthen communication and cooperation, learn from each others' experience, and collectively promote the development of the field of rare diseases.

5. Conclusion

This study used CiteSpace to analyze outstanding academic achievements in the field of rare diseases over the past decade. By visualizing and mapping key institutions, authors, keywords, and high-citation information, it identified research hotspots and development trends from different perspectives, providing insights for global advancement of the field of rare diseases.

However, there are certain limitations to this study. First, the literature selection process was manual, which may have introduced subjectivity in the inclusion of literature, organization of literature information, and merging of keywords, potentially leading to the loss of some relevant articles. Secondly, CiteSpace uses its in-built algorithms for keyword merging and clustering, which may not fully represent all research content within a particular cluster. Additionally, research on specific rare diseases often does not directly include the term "rare diseases," which implies that we may have excluded some studies focusing on individual rare diseases. Furthermore, owing to limitations in the CiteSpace software and CNKI database, the direct export of citation data was not possible, resulting in a lack of co-citation analysis for Chinese literature in this study.

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Original Article

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A bibliometric study of rare diseases in English and Chinese databases from 1985 to 2024 based on CiteSpace

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SUMMARY: This study utilizes CiteSpace (version 6.2.R3) to visually analyze literature related to rare diseases, summarizing the current research status and hotspots in the field. The goal was to provide broader perspectives and references for researchers in rare diseases. A comprehensive search for relevant literature in the rare diseases domain was conducted through the China Knowledge Network (CNKI) and Web of Science (WOS), spanning the years 1985 to 2024. Then, CiteSpace software was utilized to create a visual map of the annual publication volume, authors, institutions, keywords, and other content. After screening, 2,293 Chinese and 2,262 English articles were included in the study. Over the last several decades, the diagnosis and treatment of rare diseases have been a common research focus in both China and foreign countries, but there is a significant research depth and breadth gap. In China, there is a shortage of core authors and high-quality literature, and the level of collaboration among research teams is significantly lower compared to the robust international cooperation between authors and institutions. High-frequency and central keywords in the field include "orphan drugs", "children", and "genetic mutations", reflecting research hotspots in this domain. Research on rare diseases has been increasing annually, with key directions focusing on orphan drug development, novel therapeutic agents, genetic therapies, and healthcare security. In the research field of rare diseases, emphasis should be placed on early detection, early prevention, and early treatment. The application of genetic diagnostic techniques in clinical practice will have a broader prospect. This will be one of the direction for future research in this area.

Keywords: rare disease, visual analysis, knowledge map, CiteSpace

1. Introduction

Rare diseases are distinguished by their uncommon occurrence or low incidence rates. There is a lack of uniformity globally in the criteria used to define the incidence of rare diseases, with no universally accepted standard currently in place. The World Health Organization (WHO) categorizes rare diseases as those with a prevalence ranging from 0.65 to 1 per 1000 individuals (*1*). In the "*China Rare Disease Definition Research Report 2021*", published in 2021, China has established specific criteria for identifying rare diseases, proposing that a condition be classified as rare if it meets any of the following thresholds: a neonatal incidence rate below 1/10,000, a prevalence rate below 1/10,000, or a total affected population less than 140,000 (*2*). Rare diseases are mostly caused by specific gene mutations, often involving multiple organs and systems of the human body, showing a chronic, progressive, and consumptive development. They are characterized by a low incidence rate for individual diseases and a small proportion of the patient population (*3*). This results in a multitude of interrelated issues in the diagnosis and treatment of rare disease patients, including the intricacy of diagnosing these conditions, a high incidence of misdiagnosis, and a low prevalence of standardized therapeutic approaches (*4*). Additionally, the development of orphan drugs is marked by significant costs, with only a limited number of these medications successfully making it to the market.

Due to the enormous population base in China, the total number of patients with rare diseases is expected to exceed 20 million, creating a situation where "rare diseases are not uncommon" (*5*). These patients urgently

need effective diagnostic and treatment methods. Therefore, Chinese scholars attach great importance to rare disease research, investing more resources and funds in this field to bring about new discoveries and treatment strategies. This not only aids in promoting the government's formulation of public health policies targeting rare diseases, but also ensures the protection of patients' rights and interests. In addition, it can facilitate global cooperation in rare disease research, enhancing China's academic influence in this field. Systematically organizing and summarizing the literature related to rare diseases, and encapsulating research progress, current status, hot topics, and potential trends in the field of rare diseases plays a positive role in guiding researchers in selecting the right research direction and promoting long-term development of the field. The WOS and CNKI are the most comprehensive core journal indexing databases both in China and globally. They can help identify trends in scientific research from different linguistic and national perspectives. Based on this, this study uses CiteSpace software to conduct a bibliometric analysis of research related to rare diseases from different perspectives. The study will analyze the research in the field of rare diseases and draw a knowledge map, providing valuable references for rare disease research in China.

2. Data sources and research methods

2.1. Literature retrieval and data screening

A Chinese literature search was conducted on CNKI using the search query "rare disease" or "infrequent disease", covering the period from January 1985 to November 2024. This search yielded a total of 4,176 original Chinese articles. An English literature search was performed using the WOS database with the search query "rare disease" or "rare diseases", covering the same period from 1985 to 2024, resulting in a total of 4,981 original English articles.

To ensure the quality of the literature retrieval and the scientific validity of subsequent bibliometric analysis, data screening was conducted. All duplicate entries were removed, and editorial materials, book chapters, letters, and other types of literature were excluded. Additionally, any literature with incomplete or missing author information was excluded. After multiple rounds of screening, 2,293 Chinese literature records and 2,362 English literature records were retained (Figure 1).

2.2. Research methods

The search results from CNKI were exported in "Refworks" format, whereas the results from WOS were exported using "Plain Text - Full Record and References" as the data source, information on authors, research institutions, countries, publication years, and keywords

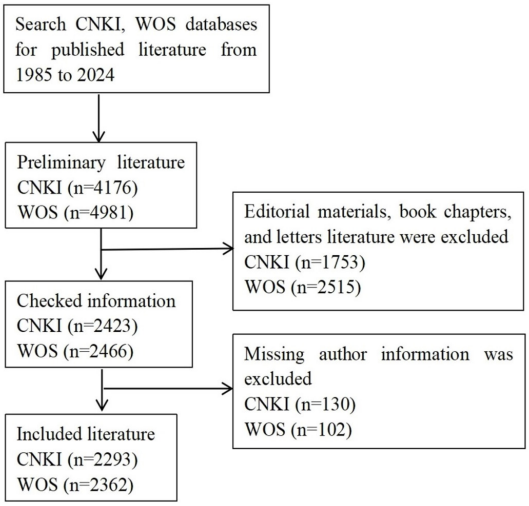

Figure 1. Flowchart of literature search and data incorporation.

were included. CiteSpace software was then used for visual analysis. The parameter settings include a "Time Slicing" span from "1985.1" to "2024.11" and a "Years Per Slice" of 1, with node types selected as author, institution, and keyword for analysis. Cosine similarity was used for connection strength, and the Pathfinder method was employed for pruning.

3. Results

3.1. Statistics of article publication volume

Through the statistical analysis of the annual publication volume, we can clearly understand development status and future trends of a specific field in China and other countries. The overall trend of the annual publication volume in the field of rare diseases shows a wave-like upward trend, since 2012, this trend has become increasingly evident. According to the CNKI database, the number of publications has risen from 47 in 2012 to 240 in 2023. Similarly, the WOS database reveals an increase of 174 articles between 2012 and 2023. Despite minor fluctuations in certain years, the upward trend in research output is apparent. It's important to note that our data for 2024 is incomplete, as we haven't included publications from November 2024 onwards, which might make the 2024 figures seem lower in comparison. Overall, rare diseases have become a significant area of research over the past decade, and it is crucial for modern scholars to continue investing in this field (Figure 2).

3.2. Distribution of countries/regions

We used CiteSpace to produce a country/region co-occurrence map (Figure 3). The map consists of 90 nodes and 902 connected lines with a network density of 0.2252, indicating that 90 countries/regions contribute to the field for rare diseases. In the WOS database, the country

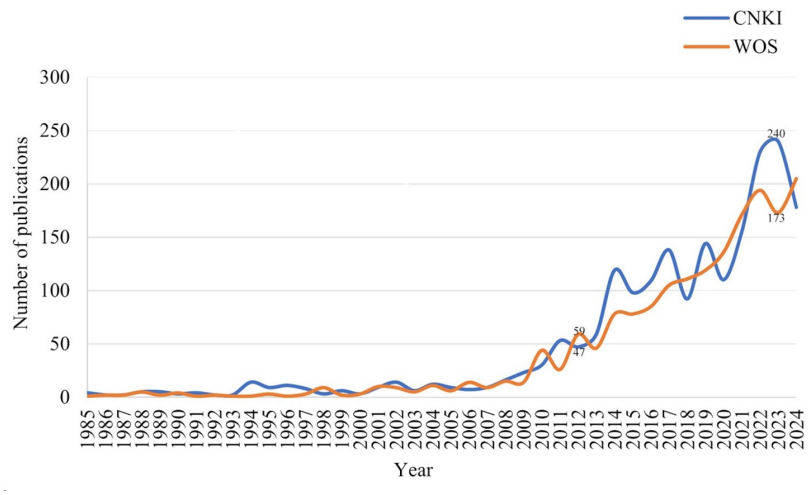

Figure 2. Annual number of publications for rare disease-related studies.

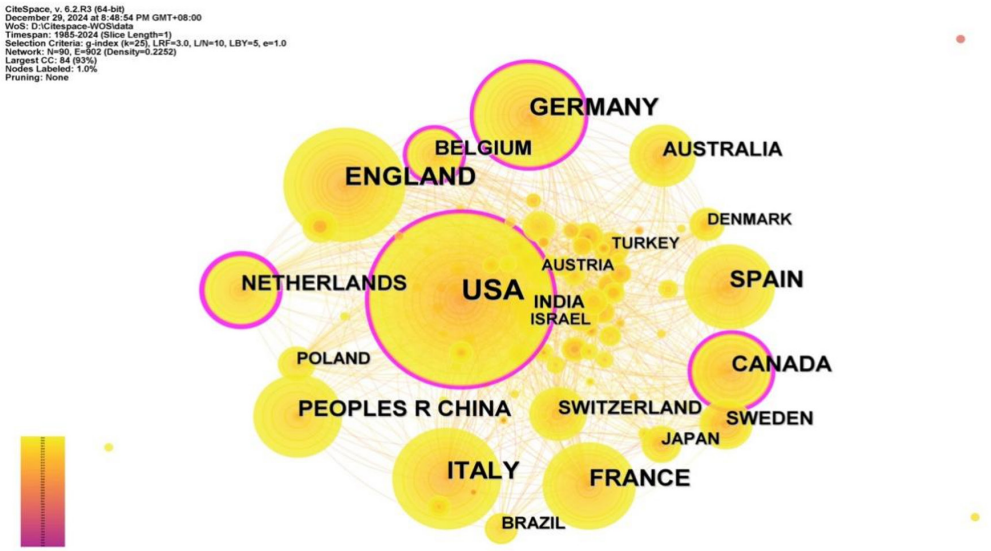

Figure 3. Network of collaborating countries.

with highest number of publications is the United States, with 1,518 related papers. Second is the United Kingdom (719 articles), followed by Italy (592 articles), Germany (492 articles), and China is ranked relatively lower (201 articles). There is a significant gap between the United States, which ranks first, and other countries, which indicates that the United States also occupies a core position in the field of rare disease research (Table 1). The purple nodes in the co-occurrence map indicate intermediary centrality above 0.1, with the Netherlands (0.22), Belgium (0.14), Germany (0.1), Canada (0.1), and the US (0.11) playing a "bridging" role in the field. Considering a combination of the volume of publications and intermediary centrality, the US and the UK have contributed the most to the field of pharmacovigilance for rare diseases. From the perspective of distribution of publishing journals, the United States and other developed European countries occupy a dominant position (Table 2).

3.3. Institution analysis

Using CiteSpace6.2.R3 software for institutional analysis, the distribution of publishing institutions is displayed (Figure 4, A and B). Nodes in the maps represent publishing institutions, with lines between nodes indicating collaborations between different institutions. Thickness of the lines reflects the degree of collaboration. The visual map of publishing institutions in the CNKI database includes a total of 360 nodes and 117 lines, resulting in a network density of 0.0018. The institution with the highest number of publications in China is Peking Union Medical College Hospital, Chinese Academy of Medical Sciences, with a total of 162 articles (Table 3). As a top medical and research center in China, it collaborates with the Institute of Basic

Table 1. The top ten countries/regions with the most publications quantity and betweenness centrality

Rank	Publications	Countries	Centrality	Countries
1	1518	USA	0.22	NETHERLANDS
2	719	ENGLAND	0.14	BELGIUM
3	592	GERMANY	0.11	USA
4	492	ITALY	0.1	GERMANY
5	491	FRANCE	0.1	CANADA
6	377	SPAIN	0.09	POLAND
7	318	CANADA	0.08	FRANCE
8	230	NETHERLANDS	0.08	AUSTRALIA
9	201	CHINA	0.06	ENGLAND
10	174	AUSTRALIA	0.06	ITALY

Table 2. Top 10 journals by publication volume

CNKI		WOS	
Publication Title	Number of publications	Publication Title	Number of publications
Pharmaceutical Economy Newspaper	82	Value in health	82
Journal of Rare and Uncommon Diseases	78	Orphanet Journal of Rare Diseases	78
China Hospital CEO	67	European Journal of Human Genetics	67
Chinese Journal of New Drugs	64	Molecular Genetics and Metabolism	64
International Journal of Pharmaceutical Research	54	American Journal of Gastroenterology	54
Journal of Clinical Pediatrics	51	American Journal of Respiratory and Critical Care Medicine	51
Chinese health	48	Advances in Experimental Medicine and Biology	48
Health For Everyone	45	Expert Opinion on Orphan Drugs	45
Health News	40	Chest	40
Progress in Pharmaceutical Sciences	40	International Journal of Environmental Research and Public Health	40

Medical Sciences of the Chinese Academy of Medical Sciences, Tsinghua University, and other prestigious institutions to jointly establish the National Key Laboratory for Difficult and Severe Diseases and Rare Diseases, as well as the Lymphangioleiomyomatosis/ Tuberous Sclerosis Complex Rare Disease Special Fund, collectively promoting research on rare diseases in China. In contrast, the visual map of publishing institutions in the WOS database comprises 280 nodes and 478 lines, with a network density of 0.0122. The institution with the highest number of publications overseas is the National Institute of Health and Medical Research. The institution was established in 1964 and boasts abundant research resources and a top-notch research team. These maps reveal a stark contrast in the collaborative landscape of rare disease research: institutions from other countries exhibit a high level of connectivity, whereas Chinese research institutions appear more isolated with limited collaborative efforts. This disparity underscores the imperative for fostering stronger international cooperation in this field.

3.4. Author analysis

The author's co-occurrence map visually depicts the core researchers in the field and the intensity of their collaborations. Each node represents an author, while the links between authors indicate collaboration. The color palette of nodes and links visually depicts starting year of collaborations, with maroon marking the beginning in 1985 and progressing through various hues to yellow for the year 2024. The size of each node is directly proportional to number of co-authors associated with each author, while the size of the node's circle reflects total number of publications by that author. The lines connecting nodes illustrate degree of collaboration between authors.

In the map, collaborative groups in the field can be identified. In the Chinese literature, the primary collaborative group is represented by Shuyang Zhang and Bo Zhang, with a total of 436 nodes, 308 connections, and a network density of 0.0032 (Figure 5A). They are all focused on the field of rare diseases in China, conducting research on pathogenesis of rare diseases and development of new drugs. In the English literature, Taruscio D, Graessner H and Boycott KM were the top three authors in terms of publication volume. The three authors constituted the center of the collaboration network, with a node count of 413, a connection count of 394, and a network density of 0.0046 (Figure 5B). These three experts have all made significant contributions to the field of rare diseases. The top ten authors are ranked in order of frequency of appearance in the literature (Table 4).

3.5. Keyword analysis

Figure 4. A network knowledge map of institutions in the field of rare diseases obtained using CiteSpace6.2.R3 software based on the CNKI database (A) and WOS database (B), respectively.

3.5.1. Keyword co-occurrence analysis

Pathfinder algorithm along with pruning of merged and sliced networks were selected in the software to obtain the co-occurrence graph of rare disease keywords by analyzing keywords from the literature in the WOS and CNKI databases separately (Figure 6, A and B). Keywords serve as a high-level summary of core topics and content of the literature. This graph reflects the frequency of occurrence and research hotspots of keywords in the field (*6*). The size of the squares in the graph indicates the frequency of keyword occurrence, with larger squares representing a higher frequency of central keywords. The CNKI database shows that the top five high-frequency keywords in China are: rare diseases (frequency = 675), orphan drugs (frequency = 164), rare drugs (frequency = 61), diagnosis (frequency = 45) and medical insurance (frequency = 40). The top five foreign high-frequency keywords are: rare diseases (frequency = 449), rare disease (frequency = 282), diagnosis (frequency = 110), orphan drugs (frequency = 92) and children (frequency = 87). It is clear that orphan medications, diagnostic methodologies, and therapeutic approaches are shared priorities in the realm of rare disease research. Centrality is a key indicator for analyzing the importance of keywords. Nodes with a centrality higher than 0.1 are regarded as key nodes. Their centrality is organized in descending order, with comprehensive details provided in Table 5. It can be learned that the centrality ranking first in the CNKI database is "rare medications", followed

Table 3. Top 10 major institutions for rare disease Chinese and English Databases

CNKI		WOS	
Publication institution	Number of publications	Publication institution	Number of publications
Peking Union Medical College Hospital, Chinese Academy of Medical Sciences	162	Institut National De La Sante Et De La Recherche Medicale	220
China Pharmaceutical University	122	University Of London	169
Beijing University	56	Assistance Publique Hopitaux Paris APHP	166
Shenyang Pharmaceutical University	50	National Institutes Of Health NIH USA	152
Shandong University	41	Universite Paris Cite	133
Fudan University	37	Harvard University	125
National Medical Products Administration	36	University College London	177
Huazhong University Of Science And Technology	32	Ciber Centro De Investigacion Biomedica En Red	108
Peking Union Medical College Hospital	28	Ciberer	95
Tsinghua University	25	University Of Toronto	89

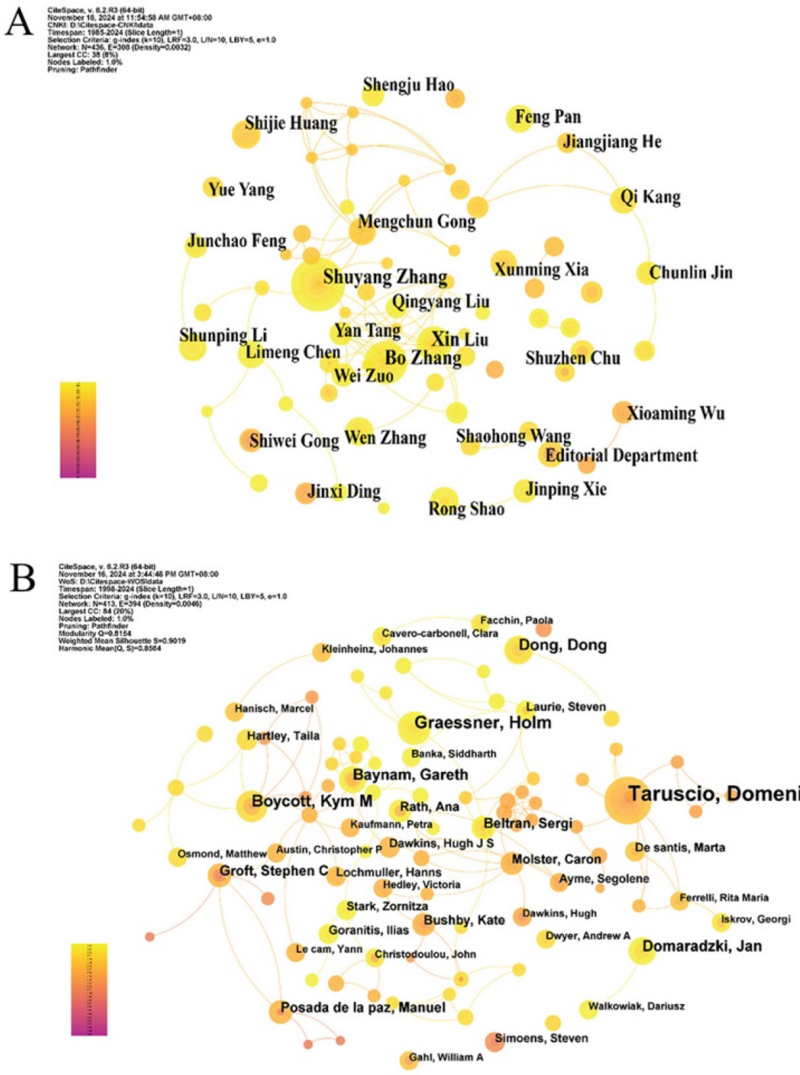

Figure 5. A network knowledge map of authors in the field of rare diseases obtained using CiteSpace6.2.R3 software based on the CNKI database (A) and WOS database (B), respectively.

by "etiologies", with research focusing on development of orphan drugs. In the WOS database, the centrality ranking first is "epidemiology", followed by "mutations", with research emphasis on gene therapy for rare diseases. This demonstrates the differences in research directions between China and the international community.

3.5.2. Keyword clustering analysis

Table 4. Statistics of the high-frequency authors in the field of rare diseases

CNKI				WOS			
Count	Centrality	Year	Author	Count	Centrality	Year	Author
43	0.01	2017	Shuyang Zhang	25	0.02	2012	Taruscio, Domenica
26	0	2019	Bo Zhang	13	0	2022	Graessner, Holm
18	0	2019	Xin Liu	11	0.01	2014	Boycott, Kym M
13	0	2016	Shijie Huang	10	0	2020	Dong, Dong
11	0	2022	Shunping Li	9	0	2021	Domaradzki, Jan
11	0	2019	Feng Pan	9	0.04	2017	Baynam, Gareth
11	0.01	2017	Mengchun Gong	7	0.03	2020	Beltran, Sergi
11	0	2021	Wen Zhang	7	0	2010	Posada de la paz, Manuel
11	0	2020	Rong Shao	7	0.01	2010	Groft, Stephen C
10	0	2022	Liming Chen	6	0.04	2014	Bushby, Kate
9	0	2017	Xunming Xia	6	0	2017	Rath, Ana
9	0	2018	Qi Kang	5	0	2010	Simoens, Steven
9	0	2010	Editorial Department	5	0	2023	Goranitis, Ilias
8	0	2022	Jinping Xie	5	0	2008	Ayme, Segolene
6	0	2023	Yan Tang	5	0.01	2023	Stark, Zornitza

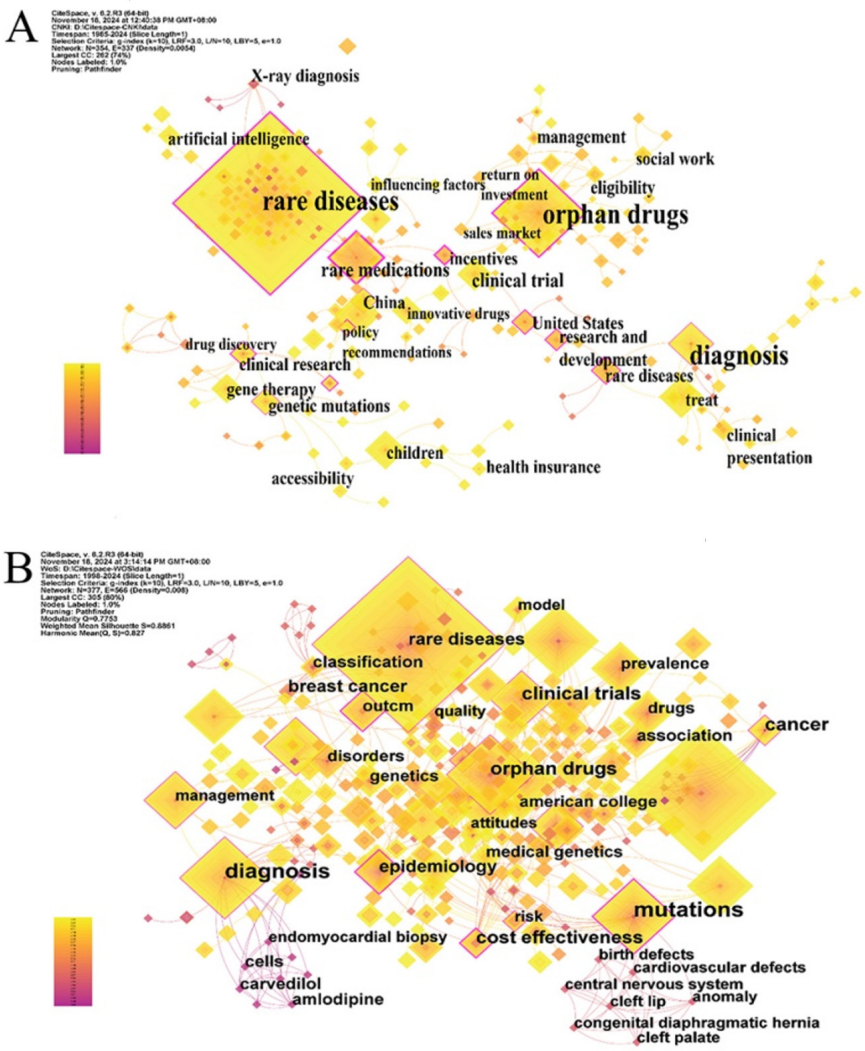

Figure 6. A network knowledge map of keywords in the field of rare diseases obtained using CiteSpace6.2.R3 software based on the CNKI database (A) and WOS database (B), respectively.

Table 5. Highly centralized keywords in the field of rare diseases

CNKI				WOS			
Count	Centrality	Year	Keywords	Count	Centrality	Year	Keywords
61	0.78	2002	rare medications	25	0.32	2008	epidemiology
6	0.6	2007	incentives	85	0.28	2005	mutations
675	0.53	1990	rare diseases	11	0.28	2010	Cost-effectiveness
164	0.42	2010	orphan drugs	9	0.19	2009	design
13	0.3	2004	United States	110	0.18	2000	diagnosis
9	0.27	2011	Research and development	54	0.16	2007	clinical trials
4	0.26	2022	medication management	37	0.16	2010	gene
15	0.24	2002	rare diseases	92	0.13	2008	orphan drugs
5	0.24	2008	new drug	54	0.12	2012	quality of life
14	0.23	2005	clinical research	52	0.12	2012	management
45	0.19	2001	diagnosis	14	0.12	1999	cancer
15	0.15	2011	genetic mutations	449	0.1	2008	rare diseases

Keyword clustering analysis was conducted using the natural logarithm method (LLR) within the spectral clustering algorithm. The modularity value (modularity Q, Q value) serves as an indicator of stability of the clustering network, with a Q value exceeding 0.3 generally considered to indicate a significant clustering effect. The average silhouette value (weighted mean silhouette, S value) represents the similarity within clustering nodes, with a typical range of 0.5 to 1.0 and a critical value of 0.5. A higher S value suggests greater similarity within clustering, making clustering more reasonable (*7*). In this study, the CNKI clustering module's Q value is 0.8561, with an average silhouette value S of 0.984, indicating clear keyword clustering and well-defined topics in the Chinese literature (Figure 7A). The WOS clustering module's Q value is 0.8154, and the average silhouette value S is 0.9019 (Figure 7B), which demonstrates a significant clustering structure and accurate, reasonable clustering results. This highlights that the clinical treatment of rare diseases and development of orphan drugs are key areas of research both in China and globally.

3.5.3. Keyword emergence analysis

Emergent terms denote keywords whose frequency increases over a specific timeframe, reflecting the research hotspot and development trends during that period. CiteSpace6.2.R3 software was employed to plot emergent terms in the study of rare diseases globally, to delve into the research trends within this field (Figure 8, A and B). The emergent strength quantifies the importance of keywords, indicating extensive citation of the term in a short time (*8*). In the visualizations, light blue bands indicate keywords that have not emerged, dark blue bands represent keywords that have appeared, and red bands signify keywords that are frequently cited during the depicted period.

In the CNKI database, the emerging keywords in rare disease research predominantly feature rare drugs, rare diseases, and orphan drugs. It is noteworthy that since 2020, there has been a growing interest among researchers in the healthcare system for rare diseases, indicating an increased focus on medical insurance aspects of treatments for these conditions. In the WOS database, focus of rare disease research is largely on clinical trials, qualitative research, patient-reported outcomes, European reference networks, and orphan drugs. Two databases both indicate that development of orphan drugs and genetic diagnosis are globally shared key research areas in the field of rare diseases. In addition, there is a focus of international attention on molecular mechanisms of rare diseases and health management of the rare disease population.

3.6. References analysis

3.6.1. Co-cited references

Highly cited references can indicate hot topics in a research field. By analyzing these co-cited references, the dynamic changes in research topics within a specific time range can be identified. Citation frequency analysis identified the top 10 most-cited English articles (Table 6). High citation frequency indicates foundational or widely referenced works in this field, providing insights into key developments and influential studies. The standards and guidelines for sequence variants jointly formulated by the American College of Medical Genetics and Genomics and the Association for Molecular Pathology were interpreted, and the effectiveness of protein-coding genes in disease screening was discussed. The article "Rare-disease genetics in the era of next-generation sequencing: discovery to translation" published by Boycott KM, *et al.* not only has a high citation frequency but also a high centrality (Table 7). It can be inferred that genetic technology, genetic medicine, and the human phenotype ontology model have significant influence and importance in the field of rare disease research.

3.6.2. Reference bursts

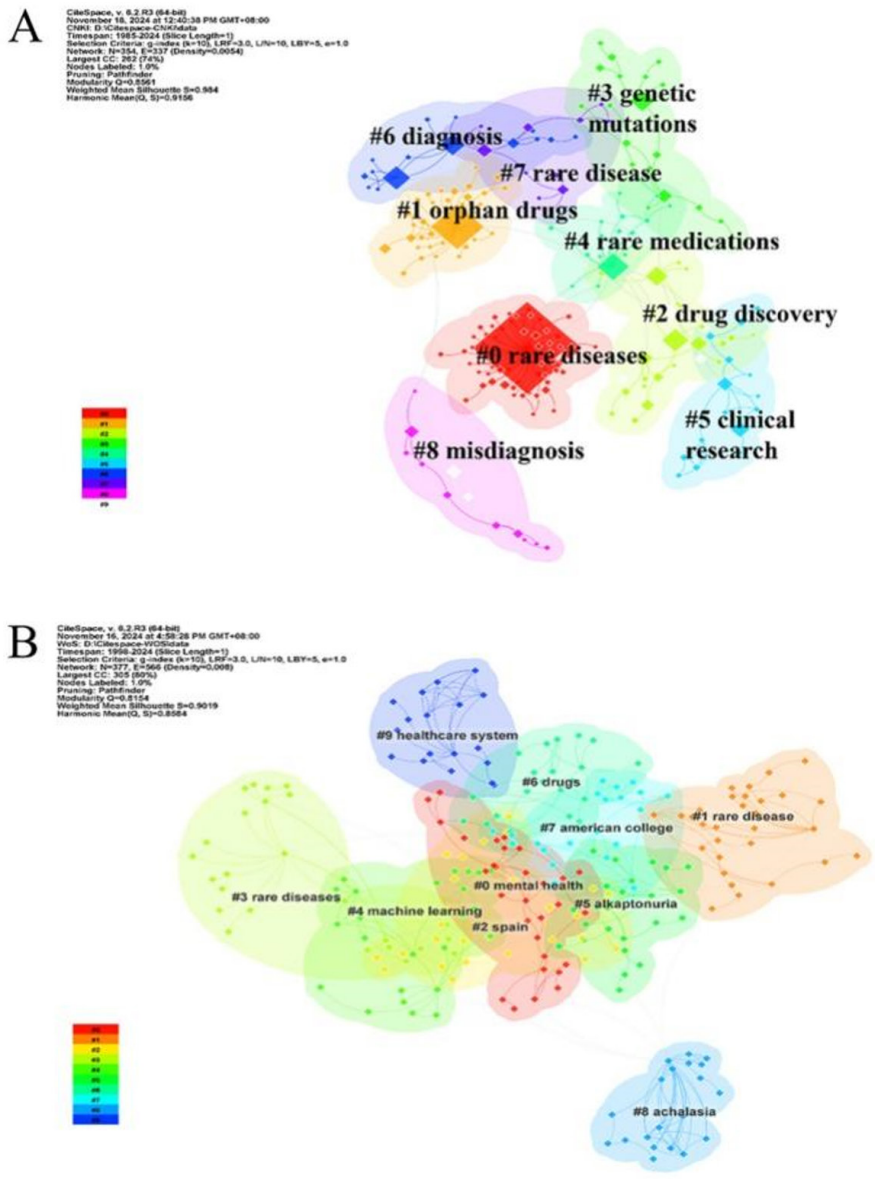

Figure 7. A clustering map of rare disease keywords obtained using CiteSpace6.2.R3 software based on the CNKI database (A) and WOS database (B), respectively.

A reference has a high burst value when it is abruptly cited heavily during a specific period, which often denotes that the research results of this reference represent innovative discoveries or new frontiers in this field (*9*). Figure 9 shows the top 25 references with the strongest burst values and their relevant information. There are three burst values exceeding 10, with citations from Wakap SN, *et al*., having a burst strength of 26.46, Haendel M, *et al.* with a burst strength of 12.29, and Betts *et al.* with a burst strength of 10.66.

4. Discussion

The rare disease research field has attracted widespread attention from scholars and achieved significant accomplishments. Here, we identified the top-producing countries, institutions, and authors. They have established representative research teams with tight internal cooperation, providing solid academic support for subsequent studies. We also determined the ten most cited influential authors and the most co-cited journals, whose publications have been widely recognized by the academic community.

4.1. Comparison of CNKI and WOS databases

In terms of the number of published papers, the United States is far ahead, with its publication volume being approximately 7.5 times that of China. Although China's paper output is still less than that of some European countries, since 2012, the number of research papers on rare diseases in the CNKI database has been continuously increasing. This trend reflects the rising attention to rare disease research in China, and there is potential for

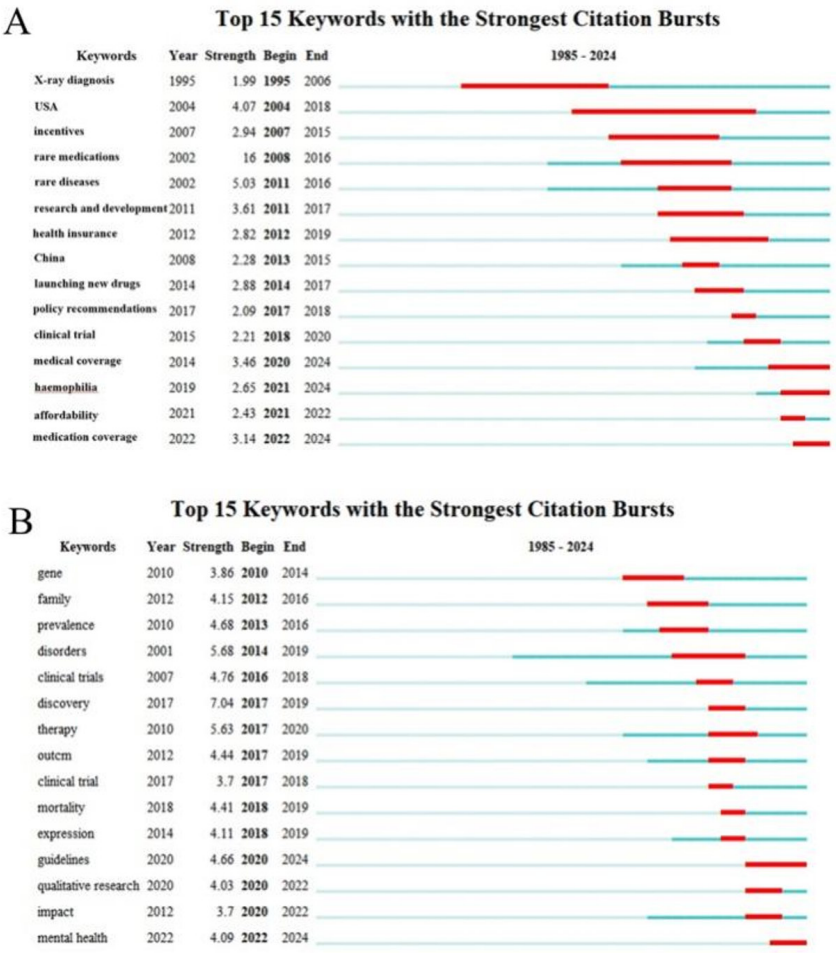

Figure 8. Top 15 keywords with the strongest bursts obtained using CiteSpace6.2.R3 software based on the CNKI database (A) and WOS database (B), respectively.

Table 6. Cited high-frequency words in the WOS database

The name of the document	First author	Year of publication	Citation frequency	DOI
Estimating cumulative point prevalence of rare diseases: analysis of the Orphanet database	Wakap, SN	2020	746	10.1038/s41431-019-0508-0
Peripartum cardiomyopathy - National Heart, Lung, and Blood Institute and Office of Rare Diseases (National Institutes of Health) workshop recommendations and review	Pearson, GD	2000	570	10.1001/jama.283.9.1183
Office of rare diseases neuropathologic criteria for corticobasal degeneration	Dickson, DW	2002	547	10.1093/jnen/61.11.935
Rare-disease genetics in the era of next-generation sequencing: discovery to translation	Boycott, KM	2013	521	10.1038/nrg3555
Why rare diseases are an important medical and social issue	Schieppati, A	2008	407	10.1016/S0140-6736(08)60872-7
Common variants at SCN5A-SCN10A and HEY2 are associated with Brugada syndrome, a rare disease with a high risk of sudden cardiac death	Bezzina, CR	1991	394	10.2307/2982708
The detection of clusters in Rare Diseases	BESAG, J	1991	380	10.2307/2982708
100,000 Genomes Pilot on Rare-Disease Diagnosis in Health Care - Preliminary Report	Peter M Visscher	2021	357	10.1056/NEJMoa2035790
On the need for the Rare Disease assumption in Case-control studies	GREENLAND, S	1991	350	10.1093/oxford journals. age.a113439
The Matchmaker Exchange: A Platform for Rare Disease Gene Discovery	Philippakis, AA	2015	346	10.1002/humu.22858

Table 7. The top 5 cited references with the highest centrality

Count	Centrality	Year	References
24	0.16	2017	Boycott KM, 2017, AM J HUM GENET, V100, P695, DOI 10.1016/j.ajhg.2017.04.003
19	0.15	2017	Köhler S, 2017, NUCLEIC ACIDS RES, V45, PD865, DOI 10.1093/nar/gkw1039
61	0.1	2019	Ferreira CR, 2019, AM J MED GENET A, V179, P885, DOI 10.1002/ajmg.a.61124
19	0.1	2013	Boycott KM, 2013, NAT REV GENET, V14, P681, DOI 10.1038/nrg3555
10	0.1	2017	Annemans L, 2017, ORPHANET J RARE DIS, V12, P0, DOI 10.1186/s13023-017-0601-9

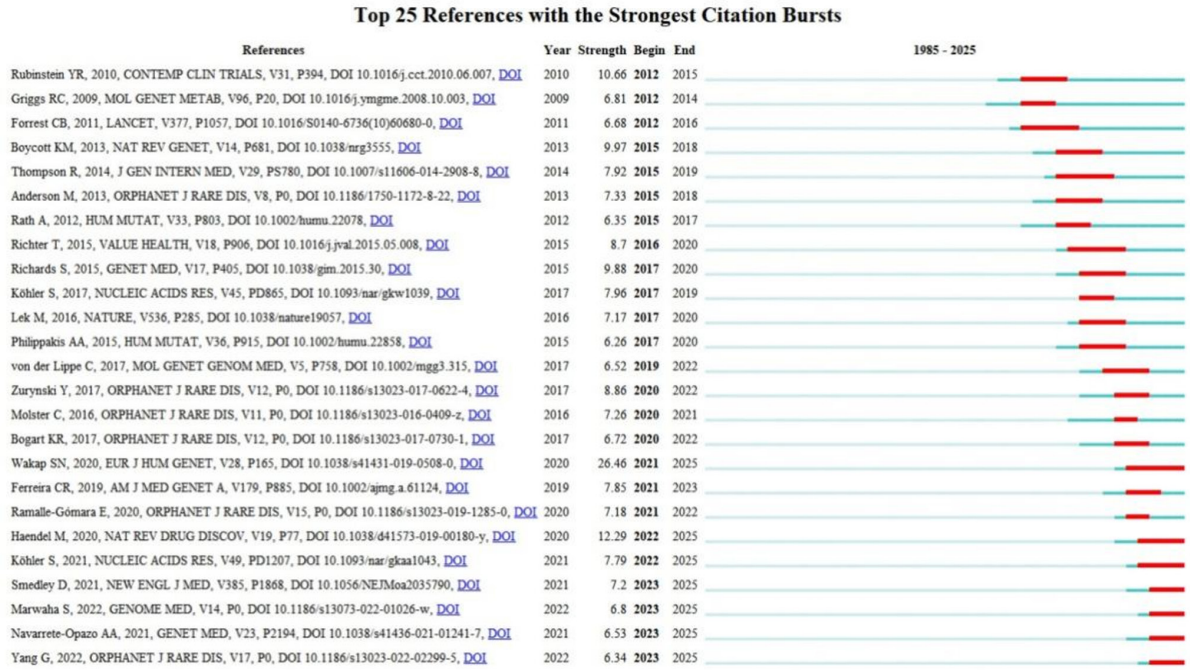

Figure 9. Twenty-five references with the strongest citation bursts.

improvement in the quality of research outcomes. The strong research capacity in economically developed countries like the United States and the European Union is due to various factors. For example, the United States is the first country in the world to formulate specific laws related to rare diseases and has established a comprehensive system for diagnosis, treatment, research, and development of orphan drugs, as well as innovative incentive mechanisms. In addition, significant financial support has been provided in these areas.

From the perspective of focus, based on the keyword frequency ranking and clustering results in CNKI, Chinese journals pay more attention to rare disease drugs, while foreign journals focus more on gene mutations and treatment. The reason behind this phenomenon may be rooted in policy differences. The United States was the first to enact specific legislation for rare diseases and has extensive experience in building a regulatory framework for these conditions. Following the Orphan Drug Act in 1983, the U.S. has continuously introduced policies like the Implementation Measures for the Orphan Drug Act and the Rare Disease Act, gradually integrating incentives for rare disease drug research and development across the entire lifecycle of pharmaceuticals. Similarly, the European Commission established the Orphan Drug Regulation in 1999, providing a legal basis for EU countries' orphan drug policies. In 2000, the European Medicines Agency formed the Committee for Orphan Medical Products to evaluate applications for rare disease drug status. These countries are pivotal in the research and manufacture of rare disease treatments. They conduct extensive genetic research, employ gene therapies, uncover disease mechanisms, and lay groundwork for new drugs and treatments. However, in China, despite existing policies that expedite approval processes, reduce taxes, and include rare disease drugs in medical insurance, most such drugs are still imported, indicating a gap compared to the U.S. and other Western countries. Hence, more time is required to enhance policies and systems concerning rare disease research, pushing China's efforts in rare disease prevention and control to a new level.

4.2. Research hotspots in the field of rare diseases

4.2.1. Orphan drug research and development

Rare diseases, often referred to as orphan diseases,

have led to the term orphan drugs for those used in their prevention, diagnosis, and treatment. The complexity and diversity of these diseases, coupled with the limited understanding and small patient populations for each condition, make the development of orphan drugs particularly challenging, lengthy, and risky. This scarcity has consistently been a major obstacle in the treatment of rare diseases. To date, over 7,000 rare diseases have been identified globally, yet only about 400 have available treatments or interventions, underscoring the pressing need for orphan drug development (*10*). In the top 25 references with the strongest citation bursts, one-third of the literature includes a focus on orphan drug research and development. The second-ranked article, "Clinical research for rare disease: opportunities, challenges, and solutions" by Grigs RC, *et al.*, mentions providing incentives for sponsors to develop promising drugs for the treatment, prevention, or diagnosis of rare diseases (*11*). The United States was an early pioneer in this field, with the Orphan Drug Act providing not only a clear definition of rare diseases, but also offering targeted research grants, tax incentives, and a 7-year market exclusivity period for orphan drugs post-launch (*12,13*). These incentives, both push and pull, have established risk-sharing mechanisms and expedited review and approval processes, substantially reducing the risks associated with orphan drug development. The European Union's Orphan Drug Regulation goes further, offering a 10-year market exclusivity period and research support, along with incentives like tax breaks and funding for pharmaceutical companies (*14*). Within the CNKI database, phrases such as "rare medications", "orphan drugs", and "new drugs" emerge with remarkable frequency, appearing hundreds of times and thus qualifying as high-frequency keywords. China, too, has introduced various policies to stimulate the development of treatments for rare diseases and is actively working to regulate and promote the growth of orphan drugs.

Different from drugs for common diseases, the development, launch, and patent approval of orphan drugs are supported by multiple international organizations and policies of many countries, leading to a much faster market penetration than non-orphan drugs (*15*). In recent years, drugs for rare diseases have accounted for over 35% of the total number of drugs approved by the FDA (*16*). Techniques such as gene therapy, antibody therapy, enzyme replacement therapy (ERT), and drug repurposing have increasingly become the focus of orphan drug research and development. The development of orphan drugs is a shared challenge and research hotspot in the global pharmaceutical industry. The emergence of new research technologies has transformed the landscape of disease treatment and offered a glimmer of hope for patients with rare diseases.

4.2.2. Genomics and genetics

Genome research can identify gene variants associated with rare diseases through whole genome sequencing or exome sequencing. Utilizing cellular and molecular genetic techniques to screen and diagnose diseases, applying genetic technology for precision treatment, exploring pathogenesis, and guiding drug development. The high-frequency keywords in the CNKI and WOS databases both show "gene" and "mutation". Gene therapy has achieved significant results in treatment of various rare diseases, such as Wiskott-Aldrich syndrome, and Fanconi anemia (*17*). In the analysis of co-cited literature, Boycott KM has two highly cited and highly prominent articles: "International Cooperation to Enable the Diagnosis of All Rare Genetic Disease" and "Rare-disease genetics in the era of next-generation sequencing: discovery to translation" (*18,19*). The articles respectively elaborate on the impact of the genome on the mechanisms of rare diseases and the use of genetic diagnosis and treatment to improve the identification rate of genes causing rare genetic diseases. These findings indicate that genetic diagnosis and treatment are a hot topic in the field of rare diseases.

In 2017, the world's first gene therapy drugs were approved for market, officially opening the era of personalized treatment and gene therapy. To date, more than 40 gene therapy drugs have been approved for clinical application worldwide, with indications including hereditary autoimmune diseases, blood disorders, neurological diseases, and solid and non-solid tumors, with treatment costs being generally high. In contrast, China's gene therapy industry is still in its infancy. So far, more than 20 products in China have entered the clinical trial phase.

In 2024, researchers from institutions such as Boston Children's Hospital, Harvard Medical School, and Dana-Farber Cancer Institute in the United States conducted sequencing and analysis of the exomes and genomes of more than 8,000 family members suspected of having rare single-gene diseases, assessing the diagnostic yield of genomic sequencing within this cohort. The study results confirmed that genomic sequencing can provide diagnostic evidence for some families affected by rare single-gene diseases, increasing the diagnostic rate for rare diseases by 8% (*20*). In recent years, China has also attached great importance to the development and application of genetic testing technology. In September 2022, the Central Special Lottery Public Welfare Fund initiated the UPWARDS project (Upgrade of Precision in Diagnostics and Treatment of Rare Diseases), led by Peking Union Medical College Hospital, with the participation of hospitals in the National Rare Disease Diagnosis and Treatment Collaboration Network. To date, 514 hospitals have joined the project, and a total of 100,500 people have been tested, playing a significant role in improving China's capabilities and level of diagnosis and treatment for rare diseases.

4.2.3. Medical insurance coverage

The challenge of rare diseases in public health has garnered significant global attention, becoming a major hurdle for public health systems in EU countries. The EU has integrated efforts to enhance the quality of life for those afflicted with rare diseases into its regulatory framework. The focus on improving patients' quality of life and expanding medical insurance coverage for rare diseases stands at the cutting edge of research in this field. The diversity of rare diseases, coupled with the small number of patients, limited market demand, and high costs of drug development, has led to a lack of interest from pharmaceutical companies in researching treatments for these conditions (*21*). Against this backdrop, numerous countries around the world have enacted orphan drug legislation over the past three decades to safeguard the rights of rare disease patients. The U.S. was the first to enact legislation related to rare diseases, establishing comprehensive systems and innovative incentives for the diagnosis, treatment, and development of orphan drugs, supported by substantial funding. The third most highly cited article, titled "The burden of rare diseases" by Ferreira CR, *et al*., mentions the health care services for patients with rare diseases (*22*). These patients face significant living expenses, making it particularly important to pay attention to their physical and mental health. Hence, establishing and enhancing rare disease drug security policies to ensure fair access to treatment for rare disease patients has become a global research priority.

In the United States, the review process for orphan drugs is, on average, at least 6 months shorter than that for conventional medications. Moreover, the US FDA typically waives or reduces the review fees for orphan drugs, and new orphan drugs can sometimes be exempt from application fees entirely (*23*). Following the EU Medicines Agency's adoption of the Orphan Medicinal Products Regulation in 2000, continuous revisions have been made, laying the groundwork for a comprehensive rare disease management system (*24*). Due to the substantial healthcare and social care system resources required for rare diseases, the fifth most highly cited article published in the Orphanet Journal of Rare Diseases, titled "Recommendations from the European working group for value assessment and funding processes in rare diseases (ORPH-VAL)" primarily discusses several decisions regarding the research and development, pricing, and reimbursement of orphan drugs, with the aim of ensuring equitable access to effective treatments for the most vulnerable populations (*25*).

Although China's articles on medical insurance for rare diseases rank lower internationally, in the CNKI database, the keyword emergence map shows that "incentive" ranks second, with "policy recommendations" also among the top 10, indicating China's significant focus on rare disease policies. In recent years, China has actively sought its own approach to rare disease research and care. For instance, the government has established a rare disease directory and integrated it into the medical insurance system, set up a national collaborative network for rare disease diagnosis and treatment to provide support, intensified drug negotiation efforts to significantly reduce medication prices, and improved a multi-tiered medical insurance system for rare diseases to alleviate the financial burden on patients (*26*). Governments, society, the market, and patients themselves are all actively exploring solutions to ensure the care and treatment of rare diseases. In May 2018, the National Health Commission and other departments jointly released the *First Batch of Rare Disease Directory*, encompassing 121 rare diseases, with 141 drugs approved for marketing in China capable of treating 53 of these conditions, thus addressing the void in China's rare disease treatment landscape (*27*). In September 2023, the National Health Commission and other departments issued the *Second Batch of Rare Disease Directory*, adding 86 new rare diseases, with 57 approved drugs available to treat 39 of these rare diseases (*28*).

4.2.4. Artificial intelligence and big data

In the most frequently cited literature, the article "Estimating cumulative point prevalence of rare diseases: analysis of the Orphanet database" published by Nguengang Wakap S, *et al.*, precisely utilizes the Orphanet database to analyze rare diseases (*29*). Orphanet (*https://www.orpha.net/*) is currently recognized as an authoritative and respected public database for rare diseases at the international level. It contains high-quality information and research results about rare diseases. Orphanet's classification integrates various types of information, categorizing rare diseases into 32 groups and initially coding them by adopting the ICD coding method. It has established a unique naming method and ORPHA coding for rare disease. Additionally, as mentioned in the 2017 article "The human phenotype ontology" by Köhler S, *et al.*, HPO is increasingly being used by different groups such as international rare disease organizations, registries, clinical laboratories, biomedical resources, and clinical software tools as a standard for phenotypic abnormalities (*30*). This will contribute to the initial efforts of global data exchange to determine disease etiology. This illustrates the importance of data collection and registration for the diagnosis and treatment of rare diseases.

With the development of technology, harnessing the powerful computing capabilities of artificial intelligence to determine the relationship between genes and disease onset, and exploring potential drug targets has become a new trend. For example, Exome Disease Variant Analysis (EVA) is a variant pathogenicity prediction and

annotation tool published in 2019 and has been applied to various rare diseases (*31*). This tool identifies causal mutations by using whole exome sequencing of trios (family members or father-mother-child), and researchers have applied it to several cases of familial diseases to demonstrate its clinical applicability. The ability of artificial intelligence to integrate and analyze data from different sources holds great potential in overcoming challenges related to rare diseases, such as low diagnostic rates, small patient numbers, and geographical dispersion.

4.2.5. Limitations of the study

Considering the purpose of this article, no visualization analysis was conducted on the research hotspots or directions for a specific rare disease. In the future, further research and analysis can be conducted in a specific direction or from a particular perspective, thereby clarifying specific research hotspots and progress in that field. Since the CiteSpace software can only support the visualization analysis of papers from a single database, although both CNKI and Web of Science, two well-known citation databases, were analyzed, there is still the possibility of biased or omitted literature analysis. Additionally, due to the limitations of the CiteSpace software and the CNKI database, it is not possible to directly export citation data, resulting in a lack of co-citation analysis for Chinese literature in this study.

5. Conclusion

This study employs CiteSpace software to conduct a systematic review of literature on rare diseases from the CNKI and WOS databases. By analyzing research institutions, authors, keywords, and highly cited documents, a visual knowledge map is created. The goal is to uncover the current status, hotspots, and trends in this field, offering valuable insights for global rare disease research. As data science and technology advance, the visualization research in the rare disease field will be further enhanced. This not only fosters international collaboration and communication, but also speeds up transition of research findings into clinical practice, ensuring more effective support and care for patients with rare diseases. Meanwhile, artificial intelligence and big data in rare diseases will empower prevention, diagnosis and treatment of rare diseases, as well as disclosing the relationship between complex diseases and rare diseases in the near future.

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Original Article

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Mitochondrial metabolic maturation in postnatal right ventricle restricted by volume overload

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SUMMARY: Right ventricular volume overload (RVVO) is a common hemodynamic abnormality in patients with congenital heart disease (CHD) and frequently leads to pathological cardiac remodeling. Our previous research demonstrated that RVVO disrupts the metabolic maturation of cardiomyocytes. Mitochondrial metabolic maturation, a crucial process in postnatal cardiomyocyte development, remains poorly understood under RVVO conditions. In this study, an mouse RVVO model was established on postnatal day 7 by creating a fistula between the abdominal aorta and inferior vena cava, confirmed by abdominal ultrasound and echocardiography. Transcriptomic analyses revealed significant downregulation of genes linked to mitochondrial metabolic maturation. Transmission electron microscopy showed impaired mitochondrial structure and maturation markers, while Seahorse assays demonstrated a marked reduction in oxidative phosphorylation rates in RVVO cardiomyocytes. These findings collectively indicated that RVVO restricted mitochondrial metabolic maturation in the postnatal RV. Targeting mitochondrial metabolic maturation could offer a promising therapeutic strategy to mitigate RVVO-induced pathological remodeling.

Keywords: volume overload, right ventricle, mitochondrial metabolic maturation, RNA sequencing

1. Introduction

Congenital heart disease (CHD) is the most common birth defect in China (*1*) and is frequently associated with a variety of complications (*2*). The right ventricle (RV) exhibits remarkable adaptability in children with CHD, functioning not only as a pulmonary ventricle under normal physiological conditions but also as a critical systemic ventricle in specific conditions. For instance, in hypoplastic left heart syndrome (HLHS) or transposition of the great arteries (TGA) following atrial switch procedures, the RV assumes systemic circulation responsibilities (*3-5*). Right ventricular volume overload (RVVO) is a common hemodynamic abnormality in children associated with CHD, and it plays a key role in determining functional status and prognosis in children with CHD (*6-8*).

Multiple studies have shown that during the early postnatal development process, cardiomyocytes undergo a metabolic transition which is critical for their maturation. From postnatal day 1 (P1) to postnatal day 7 (P7), the mouse cardiomyocytes use glycolysis as their primary energy source with a strong proliferative and regenerative potential (*3,8,9*). At P7, mouse cardiomyocytes begin the maturation process including metabolic maturation, sarcomere maturation, and electrophysiology maturation. At P21, the cardiomyocytes are fully mature and use oxidative phosphorylation as the primary energy source (*3,4*). In our previous studies, we have found that the postnatal right ventricular developmental track changed by volume overload, especially the metabolic maturation was partly interrupted by VO in a young-aged mouse RVVO model (*10-12*). There was a shift from metabolic maturation to pathologic enhanced contraction. Mitochondria, as the central hub of cellular metabolism, play an essential role in supporting the energy required by the heart (*13*). Its metabolic maturation is critical particularly during postnatal development (*14,15*). Oxidative phosphorylation and the tricarboxylic acid (TCA) cycle are the key processes in mitochondrial metabolic maturation, ensuring efficient ATP production (*16,17*). Previous studies have revealed that mitochondrial dysfunction is a hallmark of pathological cardiac remodeling (*18,19*). However, the specific impacts of VO on mitochondrial metabolic maturation in postnatal RVs remain poorly understood. Thus, explaining how VO regulating the metabolic maturation of mitochondria

in postnatal RV is critical for improving the management of CHD patients with VO.

To fully understand how VO alters the processes of mitochondrial metabolic maturation in RV, in this study, a young-aged mouse RVVO model was successfully established at P7 and followed to P21 (the endpoint of cardiomyocyte maturation). We evaluated hemodynamic changes, transcriptome, mitochondrial morphology, and mitochondrial function. Our findings provide novel insights into the mechanisms through which the RVVO restricts mitochondrial metabolic maturation, potentially contributing to relieve pathological RV remodeling.

2. Materials and Methods

Data generated in this study are available from the corresponding author upon reasonable request. All of the RNA sequencing data have been deposited in the GEO database (*https://www.ncbi. nlm.nih.gov/geo*) with accession number GSE157396.

2.1. Animal experiments

C57/BL6 mice were randomized into two groups (RVVO and control) and underwent fistula surgery or sham operation at postnatal day 7 (P7). The surgical procedure was the same as previous articles (*12*). Under general anesthesia with 4% isoflurane, a midline laparotomy was made to expose the abdominal aorta (AA) and inferior vena cava (IVC). A 0.08 mm diameter needle was used to puncture the AA through into the IVC. Then, hemostatic compression was performed for 2 min and then the abdominal wall was closed. Lidocaine was delivered locally to relieve pain. All of the procedures conformed to the principles outlined in the Declaration of Helsinki and were approved by the Animal Welfare and Human Studies Committee at the Affiliated Women and Children's Hospital of Ningbo University.

2.2. Abdominal ultrasound and echocardiography

The fistula between AA and IVC (AVF) and pulmonary artery (PA) flow were analyzed with a Vevo 2100 imaging system (Visual Sonics, Toronto, Ontario, Canada). For confirmation of an AVF, the waveform in the IVC was recorded using pulse-wave mode. To further understand hemodynamic changes in RV in both groups, the mice with patent fistula or sham operation were evaluated by echocardiography to further monitor hemodynamic changes in RV. The velocity-time integral (VTI) of the pulmonary artery (PA) blood flow, PA-velocity, RV systolic pressure, and RV stroke volume (SV) were used to evaluate RV function.

2.3. RNA quantification and qualification

The RNA sample from RV free wall was extracted with a PureLink RNA Micro Scale Kit (Life Technologies, Carlsbad, California, USA). RNA purity was checked using the NanoPhotometer®spectrophotometer (IMPLEN, Westlake Village, CA, USA). RNA integrity was assessed using the RNA Nano 6000 assay kit of the Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA). Real time-polymerase chain reaction (RT-PCR) was performed using the PrimeScript reagent kit (Takara Bio, Kusatsu, Japan). Quantitative RT-PCR (qRT-PCR) was carried out using SYBR Green Power Premix Kits (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. qRT-PCR was performed on a 7900 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), with the following conditions: 1 cycle at 95°C for 10 s, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. The primers were obtained from Generay Biotech Co., Ltd (Shanghai, China). The relative fold change was then calculated using the Δ CT method.

2.4. Library preparation

A total amount of 1 μg RNA per sample from RV free wall was used for the RNA sample preparations. Sequencing libraries were generated using the NEBNext® UltraTM RNA Library Prep Kit for Illumina (NEB, USA). Index codes were added to attribute sequences to each sample. The mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was performed using divalent cations under elevated temperature in NEB Next First Strand Synthesis Reaction Buffer (5X). First-strand cDNA was synthesized using random hexamer primers and M-MuLV Reverse Transcriptase (RNase H^-). Second-strand cDNA synthesis was subsequently performed using DNA polymerase I and RNase H. Remaining overhangs were converted into blunt ends *via* exonuclease/polymerase activities. After adenylation of the 3' ends of the DNA fragments, NEBNext Adaptors with hairpin loop structures were ligated to prepare for hybridization. To select cDNA fragments of preferentially 250-300 bp in length, the library fragments were purified with the AMPure XP system (Beckman Coulter, Beverly, MA). Then 3 μL USER Enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA at 37°C for 15 minutes followed by 5 minutes at 95°C. Then, PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers, and Index (X) Primer. Finally, PCR products were purified (AMPure XP system), and library quality was assessed on an Agilent Bioanalyzer 2100 system.

2.5. Clustering and sequencing

The clustering of the index-coded samples was performed on a cBot Cluster Generation System using a

TruSeq PE Cluster Kit v3-cBot-HS (Illumina, Shanghai, China). The sequencing was performed on an Illumina Novaseq platform, and 150 bp paired-end reads were generated.

2.6. Quality control, read mapping, and quantification of gene expression levels

Raw data in fastq format were first processed through in-house Perl scripts. Reads containing adapters, reads containing poly-N, and low quality reads were removed from raw data to generate clean data (clean reads). All of the downstream analyses were based on clean, high-quality data.

Reference genome and gene model annotation files were down- loaded from the genome website directly. The index of the reference genome was built using Hisat2 v2.0.5, and paired-end clean reads were also aligned to the reference genome using Hisat2 v2.0.5. The number of reads mapped to each gene was counted by FeatureCounts v1.5.0-p3. Fragments per kilobase of transcript sequence per million base pairs sequenced (FPKM) of each gene was calculated based on the length of the gene and read counts mapped to each gene.

2.7. Differential expression analysis

Differential expression analysis was performed using the DE- Seq2 R package (1.16.1). The resulting p-values were adjusted using Benjamini and Hochberg's approach for controlling the false discovery rate. Genes with an adjusted p-value under 0.05 found by DESeq2 were considered differential expression.

2.8. GO and KEGG enrichment analysis of differentially expressed genes

Gene Ontology (GO) enrichment analysis of differentially expressed genes was implemented by the clusterProfiler R package. GO terms with corrected p-values under 0.05 were considered to be significantly enriched. The clusterProfiler R package was used to test the statistical enrichment of differentially expressed genes in KEGG pathways.

2.9. Transmission electron microscopy

Mitochondrial morphology and sarcomere alignment were determined by transmission electron microscopy (ThermoFisher Scientific, Pittsburgh, PA, USA). The RVs removed from the mice were cut into 1 mm^3 pieces, then fixed with fresh and cold 2.5% glutaraldehyde solution overnight at 4°C. The fixed samples were dehydrated, embedded in paraffin, and sectioned into 70 nm slices. The slices were scanned with JEM-1230 (80 KV) (ThermoFisher Scientific, Pittsburgh, PA, USA).

2.10. Cardiomyocyte isolation and oxygen consumption rate (OCR) measurement

Cardiomyocytes were isolated with a Langendorff perfusion system (ADInstruments, Shanghai, China) as described previously (*9*). After perfusion, only RV free wall was removed and cardiomyocytes from RV were used for OCR measurement by Seahorse Machine.

2.11. Statistical analysis

Continuous data were expressed as means ± standard deviation. Differences were tested with Student's *t*-test if the data were normally distributed; otherwise, they were tested with the rank sum test. Values of $p < 0.05$ were considered to be statistically significant. Statistical analyses were performed using SAS software version 9.2 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Establishment of the RVVO Model in AVF mice

To investigate the effect of volume overload (VO) on the metabolic maturation of mitochondria in the postnatal right ventricle (RV), we created arteriovenous fistulas (AVFs) in postnatal day 7 (P7) mice. As shown in Figure 1, we observed blood flow at fistula with blood flow velocity over 800 mm/s (Figure 1, A and B). And the pulsatile blood flow at the abdominal aorta showed a slower flow rate than that at the fistula (Supplemental Figure S1, *https://www.irdrjournal.com/action/getSupplementalData.php?ID=231*). In contrast, no pulsatile flow was detected at the inferior vena cava, with a flow velocity of 0 mm/s (Supplemental Figure S2, *https://www.irdrjournal.com/action/getSupplementalData.php?ID=231*).

Furthermore, the pulmonary artery (PA)-velocity and PA-velocity-time integral (VTI) were examined to confirm there was VO in RV seven days after the AVF creation. Both the PA- velocity and the PA-VTI in the RVVO group were higher than in the sham group (Figure 1C). These results confirmed that the RVVO model was successfully established.

3.2. VO Downregulates Mitochondrial Metabolic Maturation Pathways in Postnatal RV

RNA sequencing was performed on RV tissues from AVF and sham-operated mice at P21 to investigate VO-induced changes in gene expression. The results showed that there were 1,907 differentially expressed genes between AVF and sham-operated mice at P21, among which 1,022 were downregulated and 855 were upregulated in RVVO groups (Figure 2A), $n = 3$. In order

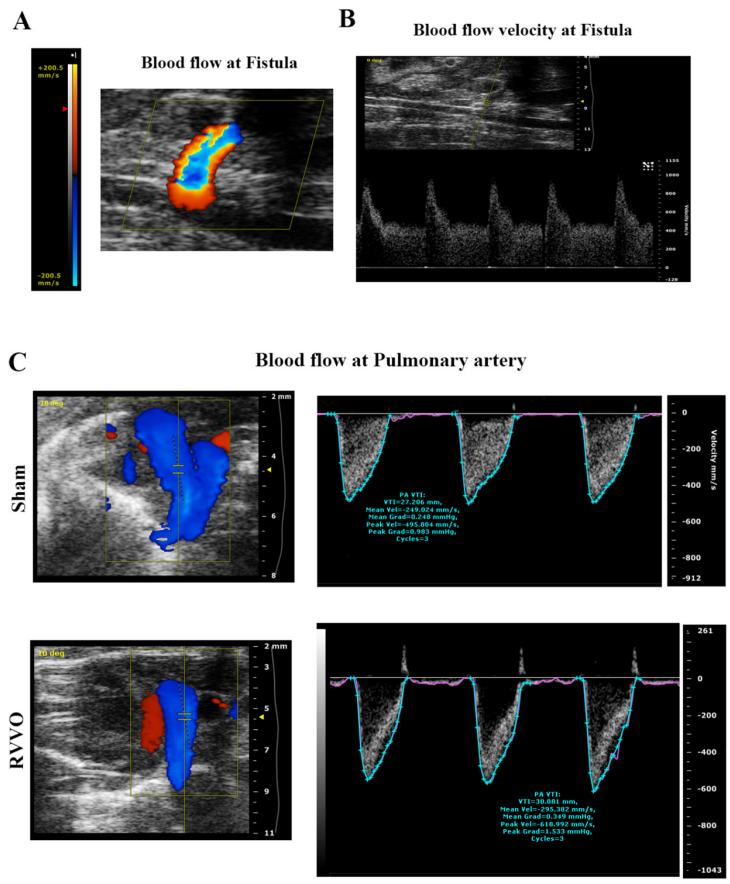

Figure 1. Construction of the abdominal aorta and inferior vena cava fistula (AVF) and verification of volume overload (VO) in the AVF group. (A) There was blood flow at fistula; **(B)** The blood flow velocity at fistula over 800 mm/s; **(C)** Representative echocardiogram of pulmonary artery (PA) velocity and velocity-time integral (VTI) in the sham and RVVO groups at P14, and the PA velocity and VTI in the RVVO group increased.

to gain a clearer understanding of the downregulation process in RVVO groups, we selected the downregulated transcripts of the RV tissues for cluster analysis. The heatmap showed that the individual mice in the same group were similar to each other but differed noticeably from the mice in the other group (Figure 2B).

Gene Ontology (GO) analysis revealed that downregulated transcripts in the RVVO group were enriched for biological processes related to energy metabolism and cellular components associated with mitochondrial structure (Figure 2C), suggesting impaired mitochondrial metabolic maturation in postnatal RV. Reactome pathway enrichment analysis further indicated that the top three downregulated pathways were mitochondrial-related (Supplemental Figure S3, *https://www.irdrjournal.com/action/getSupplementalData.php?ID=231*). To further understand the underlying mechanism by which VO modified the mitochondrial processes in postnatal RV, we applied KEGG-pathway analysis. As shown in Figure 2D, energy metabolism, oxidative phosphorylation, and citrate cycle (TCA cycle) were the most enriched terms of the KEGG pathway. This result confirmed that mitochondrial metabolic maturation was one of the most important biological processes affected by VO in postnatal RV.

3.3. Verification of RNA-seq results by examination of mitochondrial metabolic markers, morphology and respiration

To confirm that mitochondrial metabolic maturation of postnatal RV was decreased by VO, the top genes closely related to mitochondria in the gene list of RNAseq data were verified by qRT-PCR, and morphology and quantity of mitochondria were further analyzed.

As shown in Figure 3, the expression level of nine genes (Dars2, Lrpprc, Qrsl1, Mrps2, Mrpl47, Uqcc1, Gfm1, Mrps18b and Tufm), which are closely related to mitochondria, were confirmed decreased in RVVO groups, $n = 6$. Furthermore, the morphology of mitochondria was detected by transmission electron microscopy. There were fewer mitochondria in RVVO groups than in sham groups (Figure 4). In addition, the arrangement of mitochondria and sarcomeres was disordered in RVVO groups as shown in Figure 4A.

Furthermore, the effects of VO on mitochondrial respiration was analyzed by Seahorse assay. RV free wall

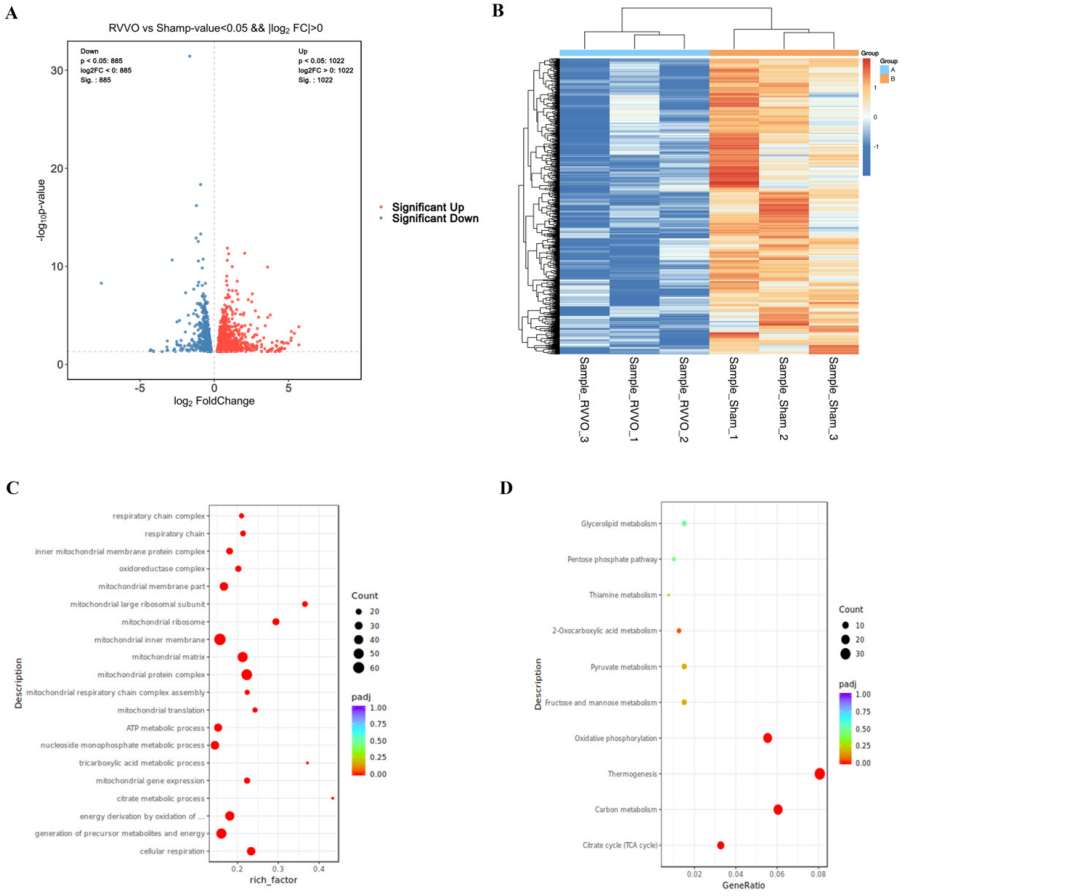

Figure 2. Transcriptome sequence revealed an enrichment of mitochondrial related genes downregulated by RVVO. (A) Volcano map of differentially expressed genes, 1022 up-regulated genes and 885 down-regulated genes in RVVO groups, $n = 3$; **(B)** The heatmap of cluster analysis of down-regulated genes (RVVO vs sham); **(C)** The top 20 GO terms of down-regulated genes were mainly related to energy metabolism and mitochondria; **(D)** The top 10 enriched terms of KEGG signaling pathways were mainly related to energy metabolism, tricarboxylic acid cycle and oxidative phosphorylation.

was removed and cardiomyocytes from RV were isolated for OCR measurement. According to the quantification of OCR levels in both RVVO and sham groups, the rate of oxidative phosphorylation was significantly decreased in RVVO group cardiomyocytes (Figure 5).

In summary, the above results confirmed that the mitochondrial metabolic maturation in postnatal RV was partly interrupted by VO.

4. Discussion

The prognosis of patients with CHDs is largely determined by RV performance, which is significantly impacted by volume overload (*6,20*). Improving our understanding of the adaption of RV to VO may help us improve our management of CHD. In our previous studies, we revealed that RVVO disrupted the metabolic maturation of cardiomyocytes. It is well known that metabolic maturation in cardiomyocytes is a hallmark of healthy cardiac development, ensuring adequate energy production to support increasing cardiac demand (*21,22*). And one of the most important processes of metabolic maturation is the change in

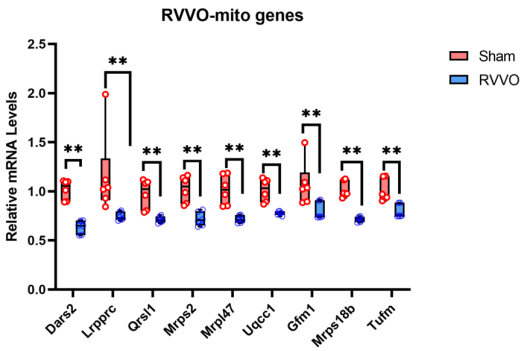

Figure 3. qRT-PCR validation of mitochondria-related genes in down-regulated gene enriched GO terms. The expression level of all mitochondria-related genes decreased in RVVO groups, $n = 6$.

mitochondria (*14,15*). However, its regulation under RVVO conditions remains poorly understood. In this study, according to our previous well-established arteriovenous fistula (AVF) model in postnatal day 7 mice (*12*), we successfully introduced RVVO groups. We also demonstrated for the first time that the impacts of VO on mitochondrial function and metabolic

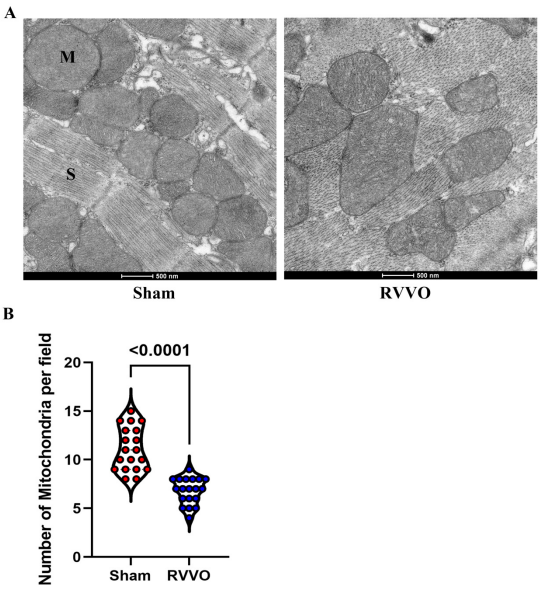

Figure 4. Transmission electron microscopy of right ventricle. **(A)** The representative images of transmission electron microscopy Of P21 RVs in sham and RVVO groups, showing that the number of mitochondria decreased and the disorder of mitochondria and sarcomeres in RVVO groups; **(B)** Quantitative analysis of mitochondria in sham and RVVO groups.

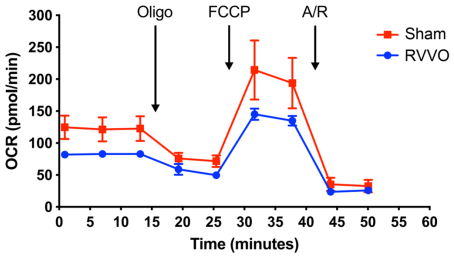

Figure 5. Quantification of OCR levels in cardiomyocytes in sham and RVVO groups. The OCR levels were decreased in RVVO group cardiomyocytes, indicating the rate of oxidative phosphorylation decreased in RVVO groups.

maturation in postnatal RVs. We observed that there was a clear shift from metabolic maturation towards pathological changes in mitochondria, characterized by reduced mitochondrial-related gene expression, deceased number of mitochondria, disordered arrangement of mitochondria and sarcomeres, and impaired oxidative phosphorylation in RVVO groups. The downregulation of mitochondrial-related genes, energy metabolism, and oxidative phosphorylation, which was indicated by transcriptomic and functional analyses, highlights the key role of mitochondrial dysfunction in this process. Furthermore, the structural abnormalities observed by transmission electron microscopy further emphasized the loss of mitochondria as a key pathological feature. These alterations not only weaken the energy efficiency of cardiomyocytes, but also may aggravate the pathological remodeling and functional decline associated with RVVO.

In summary, the current study first demonstrated that the mitochondrial metabolic maturation of cardiomyocytes in postnatal RVs was restricted by VO. This finding also provided a theoretical basis for promoting mitochondrial metabolic maturation may serve as a novel therapeutic strategy for RVVO patients. Interventions targeting mitochondrial metabolism could potentially minimize the impairments of RVVO, improving cardiac function and long-term outcomes in children with congenital heart diseases. A limitation in our study was that we only evaluated the mitochondrial metabolic maturation dysfunction in RVVO. The molecular mechanisms underlying mitochondrial dysfunction in RVVO is unknown. In the future, the molecular mechanisms and therapeutic approaches need to be explored.

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Original Article

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Risk associated circulating biomarkers S100A3 identified in congenital heart disease-associated pulmonary arterial hypertension

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SUMMARY: With improved survival rates among congenital heart disease (CHD) patients, pulmonary arterial hypertension (PAH) linked to CHD becomes more prevalent in both children and adults. PAH remains a significant contributor to morbidity and mortality in this population. Although genome-wide association studies (GWAS) have identified potential genetic variants with PAH risk and prognosis, the identification of circulating biomarkers with causal roles in CHD-PAH remains unclear. We employed the summary data-based Mendelian randomization (SMR) method, integrating expression profile data from the Gene Expression Omnibus (GEO) database related to CHD-PAH. This approach aimed to pinpoint genes causally associated with risk of CHD-PAH. We used a two-sample Mendelian randomization (MR) approach to efficiently screen for circulating proteins affecting CHD-PAH, leveraging publicly available genetic data from the UK biobank Pharma Proteomics Project (UKB-PPP) (54,219 UKB participants). Genetic determinants (cis-SNPs) of circulating proteins were used as instruments, and MR analyses assessed the influence of these proteins on CHD-PAH susceptibility in the largest PAH GWAS (2085 cases and 9659 controls). We conducted colocalization analyses to ensure shared genetic signals between circulating proteins and PAH and performed immune cell infiltration analysis to understand immune regulatory mechanisms in CHD-PAH. We found that a 1 SD increase in circulating S100 calcium binding protein A3 (S100A3) levels correlated with a reduced PAH risk (OR: 0.073, 95% CI: 0.020-0.267; $p = 0.00799$). Sensitivity analyses including various cis-SNPs, provided consistent estimates for *S100A3* (inverse variance weighted (IVW) OR: 0.085, 95% CI: 0.032-0.225; $p = 7.5 \times 10^{-7}$ and MR-Egger OR: 0.212, 95% CI: 0.013-3.376; $p = 0.387$). Colocalization analyses confirmed a shared genetic signal for *S100A3* and PAH, with a posterior probability of 99.9%. Transcriptomic investigations further highlighted *S100A3*'s protective role in CHD-PAH. Our study using SMR and GEO data identified *S100A3* as a gene associated with a reduced risk of PAH in CHD patients. Elevated circulating levels of S100A3 were linked to a reduced PAH risk, and transcriptomic evidence further supported its protective function in CHD-PAH.

Keywords: congenital heart disease (CHD), pulmonary arterial hypertension (PAH), Mendelian randomization (MR), S100 calcium binding protein A3 (*S100A3*)

1. Introduction

Congenital heart disease (CHD) is the most common congenital malformation, occurring in approximately 8 per 1,000 live births (*1*). Complex CHDs encompass a set of more severe conditions that are potentially influenced by multifactorial inheritance (*2,3*). Pulmonary arterial hypertension (PAH) associated with congenital heart disease (CHD-PAH) is a severe and complex condition that affects a significant number of patients worldwide and worsens their prognosis and quality of life. PAH is characterized by elevated pulmonary arterial pressure and vascular resistance, which can lead to right ventricular failure and ultimately, death. The survival rate for untreated PAH is dismal, with a median survival of 2.8 years from the time of diagnosis (*4*). Despite advancements in medical and surgical treatments, the management of CHD-PAH remains challenging due to its heterogeneous nature and the complexity of its underlying pathophysiological mechanisms (*1,5,6*).

Current therapeutic strategies for CHD-PAH include pharmacological treatments, such as prostacyclin analogs,

endothelin receptor antagonists, and phosphodiesterase-5 inhibitors, which improve symptoms and slow disease progression (*6,7*) but are not curative and often cause significant side effects. Surgical interventions, like defect repair or lung transplantation, carry high risks and are not suitable for everyone (*8-10*). This underscores the urgent need for novel therapeutics.

Recent studies have highlighted the involvement of circulating proteins in the pathogenesis and progression of PAH, offering promise as biomarkers for early diagnosis, prognosis, and therapeutic targets (*11*). For instance, increased levels of soluble intercellular adhesion molecule-1 (ICAM-1) have been associated with PAH severity in pediatric CHD cases (*12*). MicroRNA studies, like the identification of microRNA-27b dysregulation, also suggest roles in CHD-PAH mechanism (*13*). Despite these discoveries, the exact role of many circulating proteins in CHD-PAH remains unclear, emphasizing the need for further investigation.

Traditional statistical methods used in observational studies to investigate the association between circulating proteins and CHD-PAH risk are often limited by confounding factors and reverse causation. Mendelian randomization (MR) is a powerful epidemiological method that utilizes genetic variants as instrumental variables to infer causal relationships between exposures (*e.g.*, circulating proteins) and outcomes (*e.g.*, CHD-PAH) while minimizing confounding and reversing causation (*14*). Common variants, which can be identified through genome-wide association studies (GWAS), typically have small genetic effects (*15*) but are often used as instrumental variables. By leveraging data from genome-wide association studies (GWAS) and protein quantitative trait loci (pQTL), MR can provide robust evidence for the causal role of specific proteins in disease development.

CHD is recognized as the most prevalent birth defect (*16*) and is often associated with various complications (*2*). This study employs a two-sample MR approach to identify CHD-PAH-related proteins, validated through Bayesian colocalization and supported by lung tissue gene expression analysis. We also explore the immune regulatory mechanisms associated with CHD-PAH through analysis of immune cell infiltration. Our primary objective is to uncover novel circulating proteins that contribute to CHD-PAH pathogenesis and could serve as potential therapeutic targets.

2. Materials and Methods

2.1. Study design and data sources

We applied a two-sample MR design to identify circulating proteins associated with risk of CHD-PAH (Figure 1A). To achieve this, we used summary data from the largest genome-wide association study (GWAS) on PAH conducted among individuals of European descent (*17*), as well as protein quantitative trait loci (pQTL) GWASs from the Pharma Proteomics Project by Sun *et al.* (*18*) in the UK Biobank. Detailed methods of protein assays are described in this study (*18*).

First, the GSE113439 dataset was analyzed to identify 1,082 differential mRNA genes associated with congenital heart disease-related pulmonary arterial hypertension (CHD-PAH) in patients. After ID conversion, 1,052 genes remained, with 893 upregulated genes and 159 downregulated genes (Supplemental Table S1, *https://www.irdrjournal.com/action/getSupplementalData.php?ID=237*). Through literature search, GWAS data related to pulmonary arterial hypertension, GCST007228, was identified, which summarized data from four studies: UK National Institute of Health Research BioResource (NIHRBR) for Rare Diseases study, US National Biological Sample and Data Repository for Pulmonary Arterial Hypertension/ PAH Biobank (PAHB) study, Paris Pulmonary Hypertension Allele-Associated Risk cohort (PHAAR) study, and British Heart Foundation Pulmonary Arterial Hypertension GWAS (BHFPAH) study, as outcome data. Exposure data were obtained from deCODE plus UKB with duplicates removed, pQTL_data, $p < 5e^{-8}$, clump = 500 kb and $R^2 = 0.1$ (Supplemental Table S2, *https://www.irdrjournal.com/action/getSupplementalData.php?ID=238*); both standard two-sample method pQTL analysis and SMR method pQTL analysis were conducted. Additionally, SMR's eQTL analysis was performed for all genes. The results of the standard two-sample method pQTL analysis were corrected for p-value FDR, yielding 102 positive genes; the SMR method's eQTL and pQTL results selected genes with p_SMR < 0.05, amounting to 1,213 and 142 genes, respectively. Combining this with transcriptome data from congenital heart disease with pulmonary arterial hypertension found in the GEO database, a single regulatory gene was identified through meta-analysis (Figure 1B): *S100A3* (S100 calcium binding protein A3), and colocalization was performed using UKB protein data. The intersection of genes obtained from SMR_ eQTL [rs185078626; (Link to NCBI SNP) (*https://www.ncbi.nlm.nih.gov/snp/?term=rs185078626*)] and SMR_pQTL [rs1005436; (Link to NCBI SNP) (*https://www.ncbi.nlm.nih.gov/snp/?term=rs1005436*)] both map on *S100A3*, but they are not the same SNP; further colocalization analysis using UKB protein data revealed that only rs1005436 showed colocalization (Figure 1C), with a posterior probability of 0.99. This study reveals that the downregulation of S100A3 protein expression is closely associated with risk of PAH, consistent with the differential gene results from GEO (the expression of *S100A3* is shown in Figure 1D, $\log_2(FC) = -1.97$, adj.P.Val = 0.001). Enrichment analysis of the downregulated genes revealed that the Calcium-dependent protein binding pathway within the GO molecular function (MF) was regulated.

2.2. Ethical approval

No separate ethical approval was required due to the use of publicly available data.

2.3. Instrument selection and validation

To select genetic variants for studying the effect of CHD-PAH, we considered SNPs within the *S100A3* gene (GRCh37/hg19 chromosome 1 position 153519805–153521734) that associated with expression of the gene (*i.e.*, protein quantitative trait loci (pQTL)) in blood at genome-wide significance ($p < 5\times10^{-8}$). To ensure that the variants used as instruments in MR are not highly correlated with each other, we then ranked them in order of the P values of their associations with UKB_PPP_pQTL (*https://registry.opendata.aws/ukbppp/*) (*19*) and pruned to linkage disequilibrium correlation $R^2 = 0.1$ and distance threshold 500 kilobases (Table 1).

2.4. MR

The MR approach was based on three key assumptions (*19*). First, the genetic variants used were associated with the risk factors. With the advent of large-scale modern GWASs, genetic variants associating with exposure can be identified in large datasets (*20*). Second, the genetic variants must not be associated with

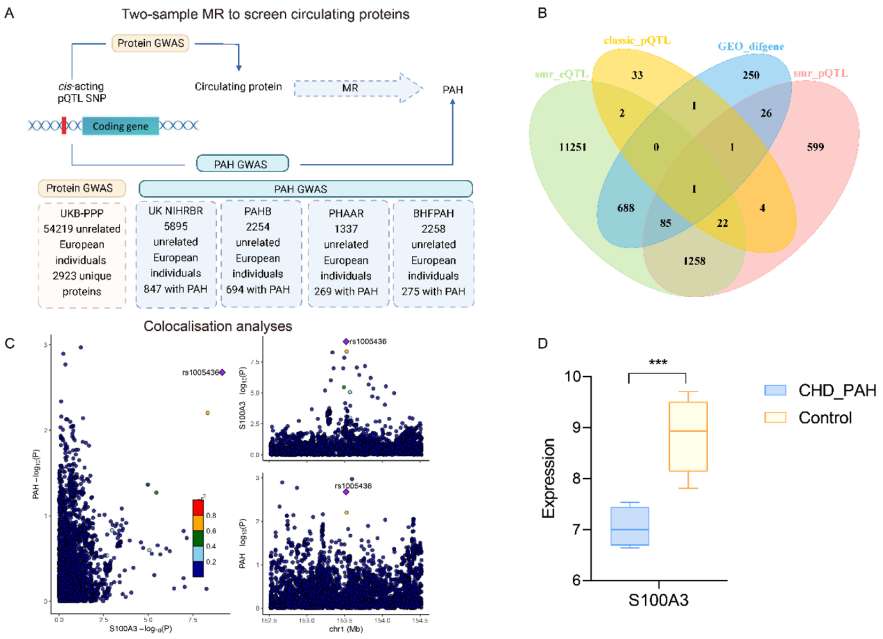

Figure 1. Overall study design. (A) Employing the classical two-sample MR and SMR methods, we obtained all gene eQTLs and pQTLs from the database and, in combination with GEO data, identified circulating protein targets for CHD-PAH; **(B)** one gene *S100A3* was identified in four data analyses; **(C)** colocalisation analyses for the target rs1005436; **(D)** *S100A3* expression significantly lower in CHD-PAH patients. *Abbreviations*: MR, Mendelian randomisation; GWAS, genome-wide association study; pQTL, protein quantitative trait loci; SNP, single nucleotide polymorphism; *S100A3*, S100 calcium binding protein A3; NIHRBR, UK National Institute of Health Research BioResource for Rare Diseases study; PAHB, US National Biological Sample and Data Repository for Pulmonary Arterial Hypertension/PAH Biobank study; PHAAR, Paris Pulmonary Hypertension Allele-Associated Risk cohort study; BHFPAH, British Heart Foundation Pulmonary Arterial Hypertension GWAS study; CHD-PAH, Congenital Heart Disease-associated pulmonary arterial hypertension; GEO dataset, GSE113439 in Gene Expression Omnibus. eQTLs, expression quantitative trait loci; GWAS, genome-wide association study; HEIDI, heterogeneity in dependent instruments; IVW-MR, inverse-variance-weighted Mendelian randomization; MR-PRESSO, Mendelian Randomization Pleiotropy RESidual Sum and Outlier; MAF, minor allele frequency; SMR, summary-data-based Mendelian randomization.

Table 1. Single nucleotide polymorphism employed as instruments for *S100A3*

rsID	Chromosome	Position (hg19)	Effect allele	Other allele	Effect allele frequency	UKB-PPP pQTL (n = 33777)					pQTL
						beta	SE	p value	R^{2a}	F statistic[b]	p value
rs1005436	1	153521932	A	G	0.128	0.07	0.011	7.18E-10	0.005	37.96	0.002
rs185078626	1	153340640	T	C	0.006	0.297	0.05	5.42E-09	0.126	34.03	0.715
rs28472359	1	153528079	C	T	0.098	0.076	0.013	4.62E-09	0.041	34.34	0.006
rs73024420	1	153497360	A	G	0.017	0.167	0.029	1.45E-08	0.015	32.12	0.067

[a]Estimates the proportion of variance in the phenotype explained by the genetic variant. [b]Measure of instrument strength. *Abbreviations*: *S100A3*, S100 calcium binding protein A3, pQTL, protein quantitative trait loci; SE, standard error. UKB-PPP, UK Biobank Pharma Proteomics Project.

confounders of the exposure–outcome relationship. A potential violation of this assumption can occur due to confounding by LD and/or population ancestry (*21*). Lastly, genetic variants must not affect the outcome, except through the exposure of interest (referred to as a lack of horizontal pleiotropy) (*22*).

Large-scale GWASs for circulating proteins (*23*) have often found that the genetic determinants of circulating proteins reside cis (in close proximity) to the encoding genes. Use of cis-acting single nucleotide polymorphisms (SNPs) for MR reduces potential horizontal pleiotropy and increases the validity of MR assumptions, because a cis-SNP strongly associated with the protein is likely to directly influence the gene's transcription and consequently the circulating protein level.

2.5. Colocalization

A threat to the validity of cis-MR analyses is confounding by linkage disequilibrium. This occurs when a variant associated with the phenotype is in linkage disequilibrium with a variant associated with the outcome, thereby producing a spurious MR association. To test the robustness of our results against such confounding, we used Bayesian colocalization analysis *via* the Coloc method (*23*) between the protein marker S100A3 and all outcomes with significant MR associations. Coloc presents evidence for five hypotheses: no causal variant for either trait, a causal variant for trait 1 but not trait 2, a causal variant for trait 2 but not trait 1, distinct causal variants underlying each trait, and a shared causal variant underlying both traits. A high posterior probability for the fifth hypothesis (> 0.8) supports the presence of a shared causal variant underlying both traits, while a high posterior probability for the fourth hypothesis (> 0.8) supports the presence of distinct causal variants underlying each trait, thus indicating confounding by linkage disequilibrium in the corresponding MR association (also referred to as horizontal pleiotropy). In the presence of a statistically significant MR association that is not a false positive finding, if the posterior probabilities for both the fourth and fifth hypotheses are < 0.8, this would suggest that the colocalization analysis is likely underpowered to discriminate whether the MR association is attributable to a shared causal variant or a confounding variant in linkage disequilibrium (*i.e.*, horizontal pleiotropy).

2.6. Sensitivity analysis

For proteins supported by MR and colocalization analyses, we conducted sensitivity analyses. In IVW (inverse variance weighted) and MR-Egger analyses, we included multiple cis-SNPs that are in weak linkage disequilibrium ($R^2 < 0.6$) with the leading cis-SNPs for candidate proteins. These analyses considered correlated variants using the MR R package (*24,25*), as the consistency of estimates could strengthen the hypothesized effects. MR-Egger allows for a y-intercept term in a random effects model. An intercept that is different from zero indicates directional horizontal pleiotropy, suggesting a violation of the third MR assumption.

2.7. Transcriptomic data in lung tissue

We first investigated *S100A3* using microarray-based transcriptomic data in CHD-PAH lung: GSE113439 (*26*). Fresh frozen lung samples were obtained from the recipient organs of 4 patients with PAH secondary to congenital heart disease (CHD) and 11 normal controls (normal lung tissue obtained from tissue flanking lung cancer resections). RNA was extracted and hybridized on Affymetrix microarrays. During the analysis, two outlier samples were identified in the normal control group and subsequently excluded to ensure the robustness and reliability of the comparative analysis.

2.8. Differential gene expression analysis and probe reannotation

We obtained preprocessed data from Gene Expression Omnibus (GEO) using the R package "GEOquery". After acquiring the expression matrix, we annotated the probesets based on the annotation profile recorded in the "AnnoProbe" package associated with the "tinyarray" package (version 2.3.2), to filter out duplicate and unannotated probes. We performed quantile normalization on the log-transformed intensities using the "normalizeBetweenArrays" function in the "limma" R package. Subsequently, we used the "limma" package to identify differentially expressed mRNAs. *P*-values were adjusted using the Benjamini-Hochberg method. Unless otherwise specified, "differentially expressed" (DE) mRNAs are defined as having an FDR < 0.05 and a $|\log_2(FC)| > 1$; $\log_2(FC) = 1$ means fold change = 2 (upregulated). $\log_2(FC) = -1$ means fold change = 0.5 (downregulated). $|\log_2(FC)|$ means absolute $\log_2$ fold change exceeds 1, meaning FC > 2 or FC < 0.5.

2.9. Functional and pathway enrichment analysis

We conducted Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis on the filtered differentially expressed genes (DEGs) using the R package "clusterProfiler" (V4.10.0) (*27*) and visualized the results. KEGG pathways and GO terms were considered statistically significant with a cut-off value of $p < 0.05$. The GO enrichment analysis consists of three components: molecular functions (MFs), biological processes (BPs), and cellular components (CCs).

2.10. Immune infiltration analysis

To assess the proportion of infiltrating immune cells in CHD-PAH gene expression profiles, we utilized the CIBERSORT (*28*) bioinformatics algorithm. This method quantifies immune infiltration by referencing the LM22 dataset, which comprises 22 immune cell subtypes and 1,000 permutations. We analyzed and visualized correlations for these immune cells with R's "corrplot" package. Differences between CHD-PAH and control samples were depicted using violin plots created with the "vioplot" package in R. Additionally, we explored the relationships between *S100A3* expression and the levels of infiltrating immune cells using Spearman's rank correlation analysis in R, with the findings graphically presented through the "ggplot2" package.

2.11. Software and preregistration

The MR analyses in this paper were conducted using the "TwoSampleMR"(version 0.6.8), "MRPRESSO"(version1.0), "locuscomparer"(version 1.0.0) R packages, as well as the SMR & HEIDI methods and software tool (*29,30*). Some data from genome-wide association studies were extracted from the OpenGWAS platform (*17*) This study was not preregistered.

3. Results

3.1. Instrument selection and validation

First, we did classic two-sample MR. After clumping, we identified 4 variants to serve as the genetic instrument for *S100A3* (Figure 2, A-C), in the MR analysis of 2,923 proteins, we found that an increase of one standard deviation (SD) in circulating S100 calcium-binding protein A3 (S100A3) was associated with a reduced risk of PAH [odds ratio (OR): 0.08, 95% confidence interval (CI): 0.020-0.267; $p = 7.51\times10^{-7}$], used FDR for *p*-value adjustment) (Figure 2A). Then we used the SMR & HEIDI methods and software tool also found lead variant predicted that a one SD increase in serum S100A3 protein levels would significantly reduce the occurrence of PAH, and the average F statistic across all variants was

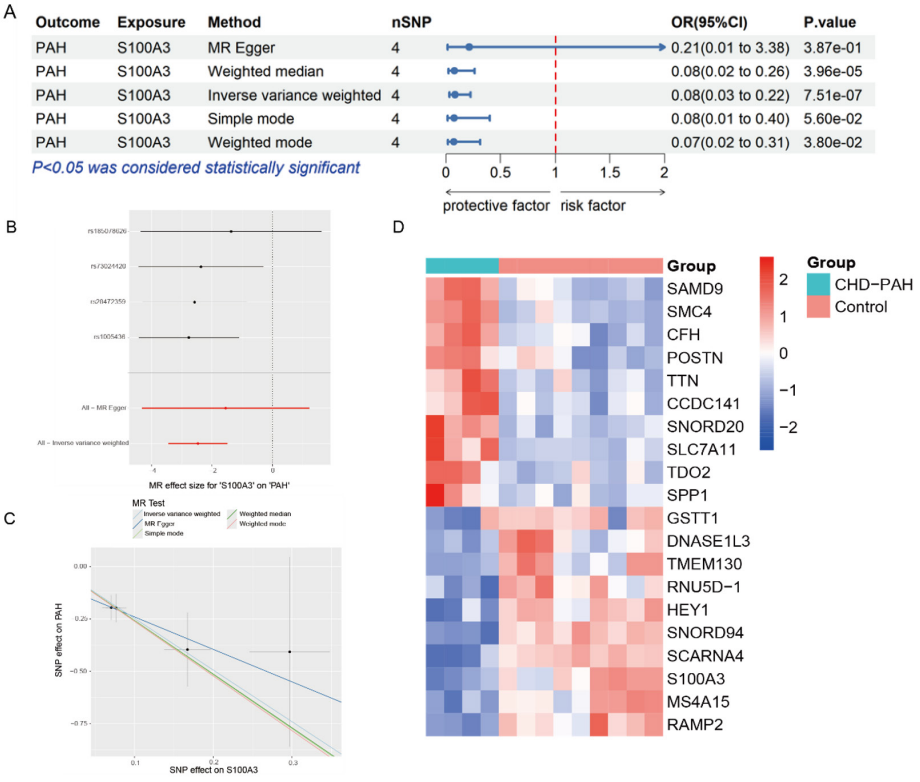

Figure 2. The association between genetically proxied serum S100A3 protein levels and pulmonary arterial hypertension related to congenital heart disease. (A) showed odds ratios are calculated per standard deviation increase in the exposure. Using the classical two-sample MR method, we obtained all potential regulatory SNPs upstream and downstream of the OID31352 marker probe encoding the S100A3 protein on human chromosome 1 and conducted MR analysis with PAH GWAS to identify positive instrumental variables, thereby confirming its potential as a therapeutic target for CHD-PAH treatment-related causal inference. **(B)** Four positive instrument SNP showed that rs1005436 was a good target. **(C)** Five methods were used for the examination, and the inverse variance weighted result was significant, suggesting that after accounting for confounding, the down regulation of S100A3 protein is causally associated with the occurrence of PAH. **(D)** displays the expression profiles of the top ten differentially expressed mRNAs, it shows that *S100A3* was the top third down regulated mRNA in congenital heart disease associated pulmonary arterial hypertension patients. *Abbreviations*: MR, Mendelian randomization; *S100A3*, S100 calcium binding protein A3; CHD-PAH, congenital heart disease associated pulmonary arterial hypertension; HEIDI, heterogeneity in dependent instruments; IVW-MR, inverse-variance-weighted Mendelian randomization; MR-PRESSO, Mendelian Randomization Pleiotropy RESidual Sum and Outlier; MAF, minor allele frequency; SMR, summary-data-based Mendelian randomization.

34 (Table 1), indicating a low risk of weak instrument bias. The positive control analysis identified a MR association in the expected direction between genetically proxied *S100A3* and pulmonary arterial hypertension ($p < 0.001$) (Figure 2, B and C). Finally, we obtained consistent results in the transcriptome data expression profiles (Figure 1D). The heat map displays the expression profiles of the top ten differentially expressed mRNAs in the lung tissue of children with pulmonary arterial hypertension associated with congenital heart disease (Figure 2D).

In two separate pQTL GWASs (deCODE+UKB, pQTL_data with duplicate proteins removed, filtered for $p < 5 \times 10^{-8}$, clumped at 500 kb with $R^2 = 0.1$) and one independent eQTL GWAS (eQTLGen_Consortium blood cis_eqtl) (Supplemental Table S3, *https://www.irdrjournal.com/action/getSupplementalData.php?ID=239*), we conducted an MR scan to identify proteins associated with CHD-PAH. After Benjamini-Hochberg correction, one candidate protein remained: circulating S100A3. A genetically determined increase of one SD in plasma S100A3 was associated with an average reduction of 63% in the risk of developing CHD-PAH (OR (95% CI) from smr_eQTL was 0.683 (0.495 to 0.872), $p = 0.371$; OR (95% CI) from smr_pQTL was 0.063 (0.040 to 0.085), $p = 0.004$) (Table 2).

3.2. Colocalization analysis

We performed a colocalization analysis between the GWAS of the candidate protein (S100A3) from Sun *et al.* (18), and the PAH GWAS to assess potential confounding due to linkage disequilibrium (LD). The SNP site rs1005436, which was significant in both the classical two-sample pQTL and SMR_pQTL (Supplemental Table S4, *https://www.irdrjournal.com/action/getSupplementalData.php?ID=240* and S5, *https://www.irdrjournal.com/action/getSupplementalData.php?ID=241*), showed excellent colocalization with PAH, with a posterior probability of 99.9% in the colocalization analyses (Figure 1C), indicating the presence of a shared signal.

3.3. Lung tissue transcriptomic data

Using microarray-based transcriptomic data from whole lung samples (GSE113439), we confirmed that *S100A3* was significantly downregulated in the transcriptome sequencing results of fresh frozen lung tissues from patients with pulmonary arterial hypertension associated with congenital heart disease compared to the control group ($\log_2(FC) = -1.97$, $p = 6.98 \times 10^{-5}$, adj.P.Val = 0.001). Further analysis of all 159 downregulated genes through GO and KEGG annotation revealed that the Calcium-dependent protein binding pathway within the molecular function (MF) category of GO was regulated (Figure 3A and 3B). Using the SMR method to obtain the risk factor corelation of CHD-PAH and PAH, the downregulation of S100A3 protein expression among the top-ranked genes reflects a causal association between these regulatory sites and the occurrence of PAH (Figure 3C), which is consistent with the data results from the GEO expression profiles (Figure1D, Figure 2D, and Figure 3C).

3.4. Immune infiltration analysis

The results of immune cell infiltration analysis showed that, compared to the control group, the expression of memory B cells, $CD8^+$ T cells, and monocyte cells was significantly increased in CHD-PAH patients (Figure 4A). Further analysis revealed that in CHD-PAH patients, the expression level of the *S100A3* gene was positively correlated with $CD8^+$ T cells, memory B cells, and NK cells (Figure 4B and 4C). These findings suggest that *S100A3* may play a complex role in immune regulation by modulating the functions of these key immune cells, thereby influencing the progression of CHD-PAH.

4. Discussion

This study addresses the essential challenge of identifying circulating proteins linked to the risk of CHD-PAH. Using a two-sample MR approach that combines GWAS and pQTL analyses, the research aimed to identify proteins associated with CHD-PAH. Bayesian colocalization analysis was employed to confirm the robustness of the findings, and differential gene expression analysis in lung tissue was conducted to further substantiate the results. The study's significant contribution is the identification of S100A3 as a key protein associated with CHD-PAH, supported by differential expression and functional pathway enrichment analyses, which elucidates potential biological mechanisms underlying the disease.

Several studies support the findings of our research, which identified S100A3 as a significant protein

Table 2. The instrument SNPs that affect pulmonary arterial hypertension at both the transcriptional and protein levels, identified through the SMR method, were mapped to the gene *S100A3*

	Gene	Method	topSNP	nsnp_HEIDI	*p*.value	OR	OR (95% CI)
mRNA	*S100A3*	smr_eQTL	rs185078626	7	0.371	0.683	0.683(0.495 to 0.872)
Protein		smr_pQTL	rs1005436	7	0.004	0.063	0.063(0.040 to 0.085)

Abbreviations: SNPs, SNP, single nucleotide polymorphism; SMR, Summary-data-based Mendelian Randomization; *S100A3*, S100 calcium binding protein A3.

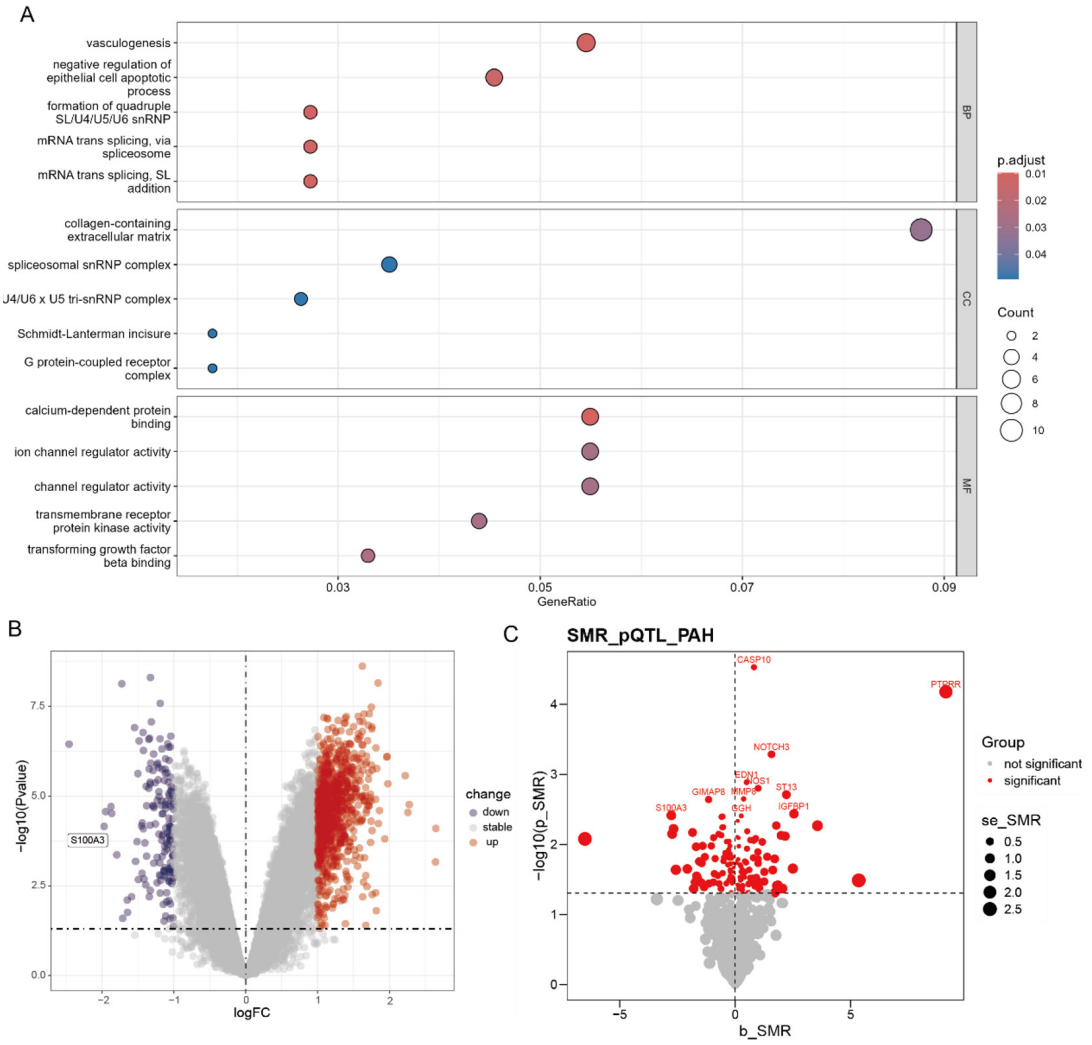

Figure 3. Analysis of the *S100A3* gene expression in CHD-PAH patients and involved pathways. (A) Enrichment analysis was performed on the downregulated genes, among which the Calcium-dependent protein binding pathway within the molecular function (MF) category of GO was found to be regulated. **(B)** and **(C)** The pQTL obtained from SMR analysis and present the volcano plot for positive exposure, showing that the downregulation of S100A3 protein expression is closely associated with the risk of PAH, consistent with the differential gene results from GEO datasect (logFC = -1.97, adj.P.Val = 0.001). *Abbreviations*: CHD-PAH , congenital heart disease associated pulmonary arterial hypertension ; GEO, Gene Expression Omnibus; logFC, log 2 fold change.

associated with CHD-PAH. For instance, the role of *S100A3* in other pathological conditions has been explored extensively. Liu *et al.* observed that *S100A3* activation is involved in tumorigenesis in colorectal cancer, suggesting its critical role in cellular processes and disease progression (*31*). This aligns with our findings where *S100A3* levels were significantly altered in CHD-PAH patients, indicating its broader relevance in various diseases. Furthermore, the study by Tao *et al.* on hepatocellular carcinoma (HCC) demonstrated that *S100A3* is implicated in tumor aggressiveness and that modulating its expression could be a potential therapeutic strategy (*32*). This supports our hypothesis that *S100A3* could be a therapeutic target in CHD-PAH as well. Additionally, a study by Gianni *et al.* highlighted the interaction of *S100A3* with RARα and PML-RARα in breast and lung cancer cells, affecting the stability and activity of these receptors (*33*). This interaction underscores the importance of *S100A3* in regulating cellular functions, which could be extrapolated to its role in CHD-PAH pathophysiology. Contrarily, some studies have reported different roles for *S100A3*. For instance, the work of Fritz *et al.* on the structural properties of *S100A3* revealed its unique calcium and zinc-binding properties, which are crucial for its function in hair cuticle formation (*34*). While this study focuses on a different biological context, it highlights the multifunctional nature of *S100A3*, which could explain its involvement in diverse pathologies, including CHD-PAH. Moreover, the research by Minato *et al.* on the evolution of *S100A3* and *PAD3* genes in mammals provided insights into the adaptive significance of these genes in hair formation (*35*). Although this study does not directly relate to CHD-PAH, it underscores the evolutionary importance of *S100A3*, suggesting its critical role in mammalian physiology, which may

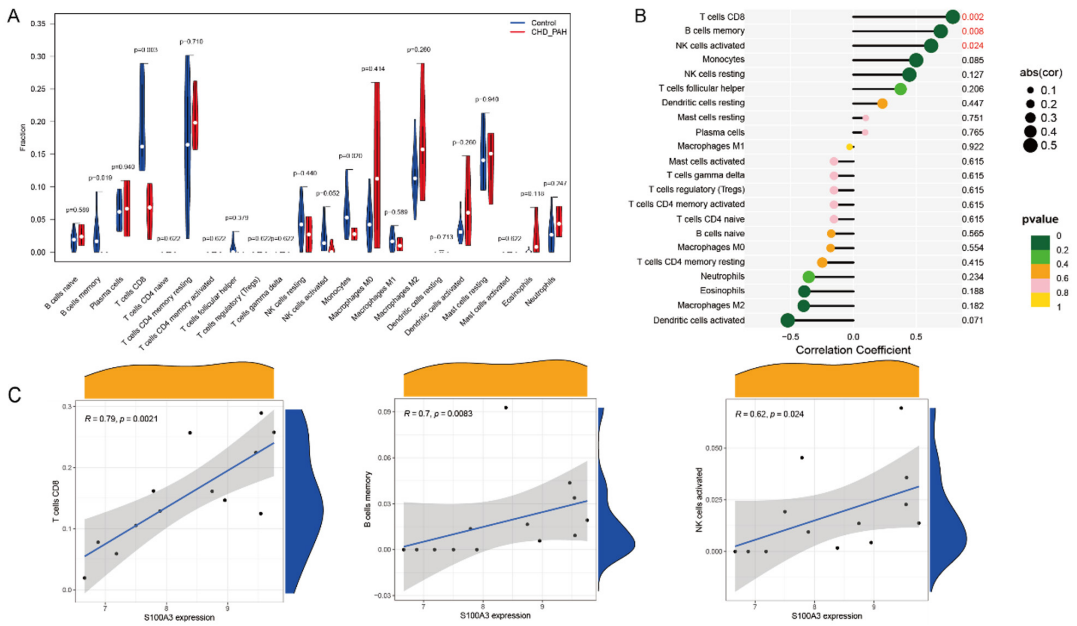

Figure 4. Analysis of the correlation between *S100A3* gene expression and immune cell profiles in CHD-PAH patients. (A) Shows the differential expression of various immune cell types between CHD-PAH patients and normal controls, with significant differences indicated by p-values. **(B)** A lollipop plot illustrating the strength and statistical significance of correlations between *S100A3* expression and different immune cell types. **(C)** Scatter plots and trend lines reveal the correlation between *S100A3* expression and specific immune cell types (*e.g.*, CD8+ T cells and NK cells), with each plot indicating the correlation coefficient (R) and *p* value.

extend to cardiovascular diseases. In conclusion, the existing literature supports our findings regarding the role of *S100A3* in CHD-PAH. The consistent observation of *S100A3*'s involvement in various diseases and its regulatory functions in cellular processes reinforce its potential as a biomarker and therapeutic target. However, further studies are necessary to elucidate the precise mechanisms by which *S100A3* influences CHD-PAH and to explore its potential in clinical applications. Despite the limited specific literature on *S100A3* in the context of CHD-PAH, the discussion can still be framed around the identified results and general mechanisms of S100 proteins and their role in cardiovascular diseases.

Our study identified S100A3 as a significant protein associated with CHD-PAH, suggesting a protective role against PAH. S100 proteins, including S100A3, are known to be involved in various cellular processes such as cell cycle progression and differentiation, which are critical in maintaining vascular homeostasis (*31,33,35,36*). The downregulation of *S100A3* in patients with CHD-PAH, as observed in our transcriptomic analysis, indicates a potential disruption in these cellular processes, contributing to the pathogenesis of PAH. The significant association between S100A3 levels and PAH risk, supported by both MR and colocalization analyses, underscores the potential mechanistic role of S100A3 in modulating pulmonary vascular remodeling. This remodeling is a hallmark of PAH and involves the proliferation and migration of pulmonary arterial smooth muscle cells (PASMCs) and pulmonary vascular endothelial cells (PVECs). The protective effect of higher circulating S100A3 levels could be mediated through its influence on PASMCs, possibly by inhibiting their proliferation and promoting apoptosis, thereby preventing vascular remodeling and subsequent PAH development. Furthermore, the genetic association of the rs1005436 SNP with PAH, as demonstrated in our pQTL and SMR_pQTL analyses, highlights the importance of genetic factors in regulating *S100A3* expression and its downstream effects on pulmonary vasculature. The high posterior probability of colocalization at this SNP locus suggests a shared genetic basis for S100A3 expression and PAH risk, providing a potential target for therapeutic intervention.

Our findings are further supported by the GO and KEGG pathway analyses, which revealed that calcium-dependent protein binding pathways are significantly regulated in CHD-PAH patients. S100A3 is a small calcium-binding protein (molecular weight ~10-12 kDa) belonging to the S100 protein family, characterized by tissue- or cell type-specific expression patterns. It contains two EF-hand calcium-binding sites, and calcium binding induces conformational changes that facilitate interactions with ligands or specific receptors. *S100A3* typically forms homodimers, heterodimers, and higher-order oligomers, and is involved in various cellular processes, including cell cycle regulation, proliferation, differentiation, migration, metabolism, cytoskeletal dynamics, signal transduction, and cell death (*36*). The dysregulation of these pathways in CHD-PAH patients could contribute to the observed downregulation of *S100A3* and its protective effects against PAH. Our study

provides compelling evidence for the involvement of *S100A3* in the pathogenesis of CHD-PAH. The protective association of higher *S100A3* levels with reduced PAH risk, supported by genetic and transcriptomic analyses, suggests that S100A3 could be a potential biomarker and therapeutic target for PAH. Further research is needed to elucidate the precise molecular mechanisms by which *S100A3* exerts its protective effects and to explore its potential in clinical applications.

In our study, using a two-sample MR approach combined with GWAS and pQTL analysis, we identified circulating S100A3 protein as significantly associated with a reduced risk of CHD-PAH. This finding is novel and robust, supported by Bayesian colocalization analysis and differential gene expression in lung tissue, which consistently showed downregulation of *S100A3* in CHD-PAH patients. Previous studies have highlighted the complexity and high risk associated with pulmonary arterial hypertension in congenital heart disease (PAH-CHD), emphasizing the need for precise biomarkers and therapeutic targets (*7,33*). The association of *S100A3* with CHD-PAH provides new insights into the pathophysiological mechanisms underlying this condition and opens potential avenues for targeted therapy. Unlike earlier studies that primarily focused on clinical and hemodynamic parameters (*37,38*), our approach integrates genetic and proteomic data, offering a more comprehensive understanding of the disease. Additionally, our results remained significant after FDR adjustment, and the SMR & HEIDI methods further validated the findings, demonstrating the reliability of our results. The identification of rs1005436 SNP as a key genetic locus associated with PAH risk through colocalization analysis underscores its potential as a genetic marker for early diagnosis and risk stratification in CHD-PAH patients.

In reflecting upon the limitations of this study, several aspects warrant consideration. First, the study primarily relies on computational methods and lacks integration with wet lab experiments, which could provide additional validation and insights into the biological mechanisms underlying the associations identified. Second, the sample size used in the analysis may be relatively small, potentially limiting the generalizability of the findings. Furthermore, the absence of clinical validation analyses means that the practical applicability of the identified proteins in clinical settings remains uncertain. Additionally, the use of multiple datasets introduces the possibility of batch effects, which could influence the results and interpretations.

In summary, this study successfully identifies circulating proteins associated with the risk of CHD-PAH, with a particular emphasis on the S100A3 protein. The integration of MR, colocalization analysis, and differential gene expression analysis provides robust evidence supporting these findings. Looking ahead, these results pave the way for further research to explore the biological mechanisms and potential therapeutic targets for CHD-PAH. Future studies should aim to incorporate larger sample sizes, clinical validation, and experimental approaches to enhance the understanding and applicability of these findings in clinical practice.

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Original Article

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Evaluating the impact of mandibular developmental abnormalities and distraction osteogenesis on swallowing function in Pierre Robin Sequence

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SUMMARY: This study aims to evaluate the relationship between mandibular developmental abnormalities and swallowing function in children with Pierre Robin Sequence (PRS). Swallowing function was assessed by a Modified Kubota Drinking Test (MKDT). Pre- and postoperative CT scans of PRS patients who underwent Mandibular Distraction Osteogenesis (MDO) were analyzed through three-dimensional (3D) digital reconstruction technology. Mandibular and airway evaluation parameters were measured, including the distance between bilateral mandibular angular, the length of bilateral mandibular ramus, mandibular notch angle (α), mandibular angle (β), mandibular body angle (γ), and the lateral and longitudinal dimensions of the posterior lingual airway. Results showed that the length of the bilateral mandibular rami and posterior lingual airway dimensions were significantly reduced postoperatively compared to controls ($p < 0.01$). After MDO, the length of mandibular rami and lateral retroglossal airway dimensions increased, α and β angles increased, while γ angle decreased ($p < 0.05$). Notably, the distance between bilateral mandibular angles, mandibular rami length, and lateral retroglossal airway dimensions had the strongest impact on swallowing score. In conclusion, mandibular width, length, and airway dimensions were closely linked to swallowing function in PRS patients. MDO effectively improved mandibular hypoplasia, improved swallowing dysfunction, and significantly enhanced quality of life for the patients.

Keywords: Pierre Robin Sequence, swallowing function, Mandibular Distraction Osteogenesis

1. Introduction

Despite China's declining fertility rate (*1*), the increasing recognition of the complex causes of rare diseases and their frequent association with other intricate conditions has brought heightened attention to these medical challenges (*2*). Pierre Robin Sequence (PRS) is a congenital disorder characterized by micrognathia, glossoptosis, and cleft palate, which collectively contribute to upper airway obstruction and feeding difficulties. According to the European guidelines, the diagnostic triad includes micrognathia, glossoptosis, and respiratory distress (*3*). Due to mandibular hypoplasia, PRS patients often experience posterior displacement of the tongue base, leading to airway obstruction, which can be life-threatening in severe cases (*4*). While progress has been made in understanding the mechanisms of airway obstruction and surgical interventions offer partial relief, the relationship between PRS and swallowing dysfunction remains poorly understood (*5*).

Children with PRS often face feeding challenges primarily due to airway obstruction caused by glossoptosis and micrognathia, which disrupt normal swallowing mechanics. Research highlights significant abnormalities in both oral and esophageal motility, including sucking-swallowing discoordination, incomplete lower esophageal sphincter (LES) relaxation, and abnormal esophageal wave patterns (*6*). These dysfunctions may elevate the risk of aspiration pneumonia. Additionally, elevated LES tone and gastroesophageal reflux further impair swallowing and respiratory function (*7*). These abnormalities in esophageal dynamics are potentially associated with gastroesophageal reflux, which exacerbates swallowing difficulties and respiratory problems. Early palatoplasty combined with submucosal release procedures has been shown to effectively reduce the incidence of upper respiratory infections and gastroesophageal reflux, thereby improving swallowing function (*8*). However, since cleft palate repair is usually performed after the

age of 8 months, persistent swallowing difficulties in the interim can result in malnutrition, which may delay neurological and overall physical development, significantly impacting the child's health. Thus, PRS patients experience multiple factors that impair swallowing function, including abnormalities in oral and esophageal motility as well as gastroesophageal reflux. Comprehensive treatment should address airway and swallowing issues to reduce complications and improve quality of life (*9*).

Mandibular Distraction Osteogenesis (MDO) is widely recognized as an effective treatment for PRS, promoting mandibular growth to alleviate airway obstruction and feeding difficulties (*10*). MDO improves health-related quality of life, particularly physiological outcomes (*11*). The use of distraction devices enables rapid mandibular advancement, which can effectively expand the airway and relieve respiratory distress within a short period (*12*). The application of 3D printing technology in MDO has demonstrated promising outcomes, facilitating surgical planning and reducing complications. While MDO has been extensively studied for alleviating airway obstruction, its effects on swallowing dysfunction remains unclear. Mandibular advancement is thought to indirectly benefit swallowing function by mitigating glossoptosis and improving airway patency (*13*). However, the precise relationship between mandibular morphological changes in the mandible and swallowing remains unclear. Despite these gaps, MDO is regarded as an effective treatment option that significantly improves PRS patients' physical health, emotional well-being, and quality of life (*14*).

This study aims to evaluate mandibular morphology following MDO and examine how these morphological changes influence swallowing function. The ultimate objective is to optimize surgical treatment strategies and lay the groundwork for integrating digital-assisted MDO to simultaneously address airway obstruction and swallowing disorders in PRS patients. By achieving these goals, the study seeks to enhance long-term quality of life for affected individuals.

2. Patients and Methods

2.1. Study population

The study was approved by the Ethics Committee at Guangzhou Women and Children's Medical Center and written informed consent was obtained from all parents or other guardians prior to enrollment (approve #: 2024307A01). Additionally, the conduct of the research complies with the Declaration of Helsinki. In this study, 15 cases of isolated Pierre Robin Sequence were randomly enrolled from patients treated at the Department of Oral and Maxillofacial Surgery. Diagnosis of PRS was made based on the clinical consensus report. Ten cases with cleft palate were randomly selected as the control group. Exclusion criteria for the study were as follows: *i*) syndromic PRS; *ii*) severe cardiopulmonary disease; *iii*) head and neck tumors or trauma leading to changes in the local anatomical structure; *iv*) laryngomalacia, brain-induced central apnoea, or mixed apnoea; and *v*) other anomalies causing airway obstruction. MDO was performed on all 30 included patients according to previously described methods (*15*), and the mandibular distraction devices were removed six months later.

2.2. Swallowing function assessment, imaging acquisition, three-dimensional reconstruction and parameters analysis

First, the swallowing function of patients was assessed preoperatively and postoperatively using a quantitative swallowing function scale, primarily based on the Modified Kubota Drinking Test (MKDT). MKDT is a clinical assessment tool used to evaluate swallowing function, particularly in pediatric patients.

Second, after computed tomography (CT) scanning, the images of preoperative and 6-month postoperative patients with PRS were collected. Mimics 21.0 image analysis software was used for reconstruction and analysis. The measurement parameters were as follows: *i*) distance between bilateral mandibular angular; *ii*) the length of bilateral mandibular ramus; *iii*) mandibular notch angle (α) formed by the anterior border of the mandibular ramus and the superior border of the mandibular body; *iv*) mandibular angle (β) formed by the posterior border of the mandibular ramus and the inferior border of the mandibular body; *v*) mandibular body angle (γ) formed by the bilateral mandibular bodies; *vi*) lateral and Longitudinal dimensions of the posterior lingual airway.

2.3. Statistical analysis

In this study, statistical analyses were conducted using SPSS software (version 22.0; IBM Corp., Armonk, NY, USA). A two-sample *t*-test was employed to assess the differences in various indices between the normal group and the preoperative group. A paired *t*-test was used to compare the indices between the preoperative and postoperative groups.

Subsequently, variables that exhibited statistically significant differences were further analyzed using multiple linear regression. A stepwise regression analysis was performed to explore the impact of the selected covariates on the swallowing score. For all statistical tests, a significance level of $p < 0.05$ was considered to indicate statistical significance. SHapley Additive exPlanations (SHAP) algorithm (*16*) and multivariate logistic regression models were also used to assess the correlation between anatomical factors and the swallowing score before and after MDO.

3. Results

3.1. Imaging of the mandible with PRS using 3D reconstruction

Imaging results reveal that children with PRS often present with a shortened and small mandible (Figure 1A, B and C), along with mandibular retraction, a significant posterior drop of the tongue base (Figure 1D), and narrowing of the posterior lingual airway (Figure 1E).

Mandibular lengthening is achieved through MDO, where a traction device is fixed at the mandibular angle to extend the length of the mandible (Figure 2A). Over six months, the device guides bone growth (Figure 2B) and helps reconstruct the jaw morphology using mimics software. It can be visually observed that the mandible is significantly elongated, improving mandibular retrognathia and alleviating tongue prolapse (Figure 2).

3.2. Assessment of swallowing dysfunction in PRS patients

Preoperative assessments using the MKDT and clinical swallowing examinations revealed moderate to severe swallowing dysfunction in PRS patients (Figure 3). Symptoms included the need for nasogastric tube feeding, frequent choking during oral feeding, and

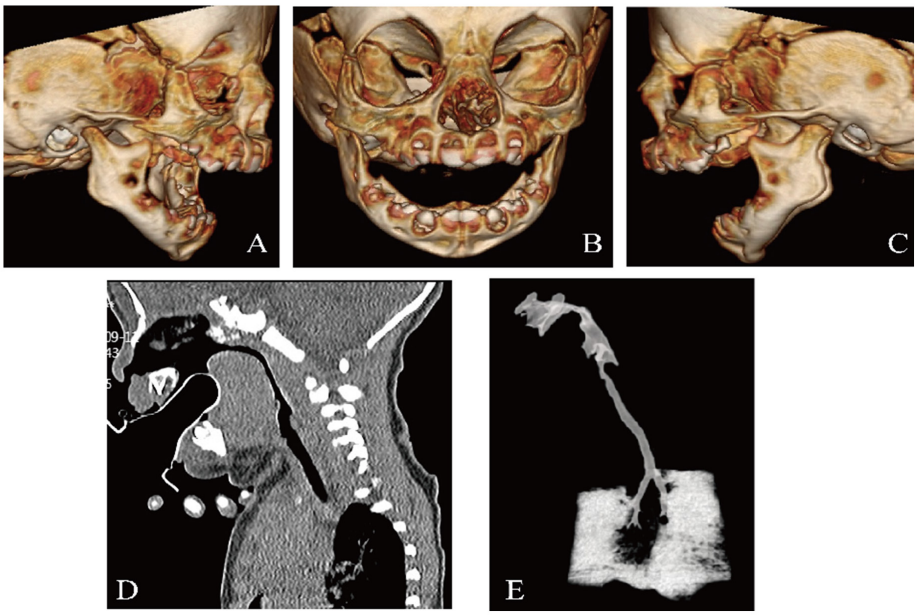

Figure 1. Imaging data collection of patients with PRS, including reconstruction of the mandible and airway. (A) Lateral view of the mandible (right side); **(B)** Frontal view of the mandible; **(C)** Lateral view of the mandible (left side); **(D)** Lateral view of the airway; **(E)** Airway reconstruction. PRS, Pierre Robin Sequence.

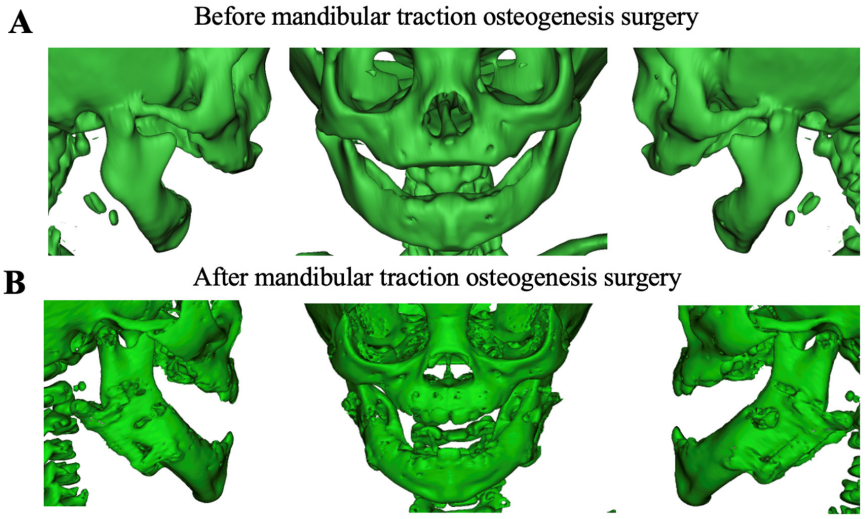

Figure 2. Changes of mandibular morphology before and after traction osteogenesis in PRS. (A) Lateral and frontal view of the mandible before MDO; **(B)** Lateral and frontal view of the mandible after MDO. PRS, Pierre Robin Sequence; MDO, Mandibular Distraction Osteogenesis.

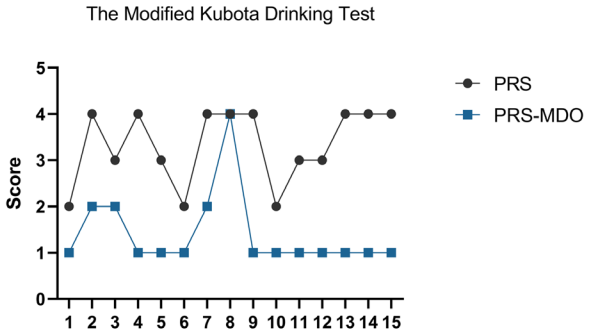

Figure 3. Modified Kubota Drinking Test Scale. Score 1: normal swallowing function, no choking or coughing observed, no changes in respiration during drinking; Score 2: mild difficulty swallowing, occasional choking or coughing observed, but no significant changes in respiration; Score 3: moderate difficulty, frequent choking or coughing, noticeable changes in respiration during drinking; Score 4: severe swallowing difficulty, inability to drink without aspiration, significant changes in respiration, often requiring interventions such as nasogastric tube feeding; Score 5: unable to swallow safely, complete dependence on alternative feeding methods (*e.g.*, nasogastric or gastrostomy tube). Each level is determined based on the child's ability to drink a specified amount of water (often 3 mL or 30 mL) in one go, while observing for symptoms like choking, coughing, or changes in breathing patterns. PRS-MDO, PRS after MDO.

weakened tongue and soft palate elevation strength, although masticatory muscle function was generally preserved. Postoperative assessments after MDO surgery demonstrated significant improvement in these symptoms.

3.3. Evaluation of mandibular morphology with PRS

The results showed that both the bilateral mandibular angular length and mandibular ramus lengths were significantly smaller in the PRS group compared to the control group ($p < 0.01$). This suggest that PRS mandibles are not only shorter in ramus length but also narrower in mandibular body width, which may contribute to airway stenosis (Figure 4A).

Furthermore, the mandibular angle in the PRS group was smaller than in controls, likely due to the shorter ramus and body, while the relatively increased angle of the mandibular body might be linked to tongue retraction (Figure 4B).

After MDO surgery, there was a significant increase in the bilateral mandibular angular and ramus lengths compared to the preoperative PRS group, indicating

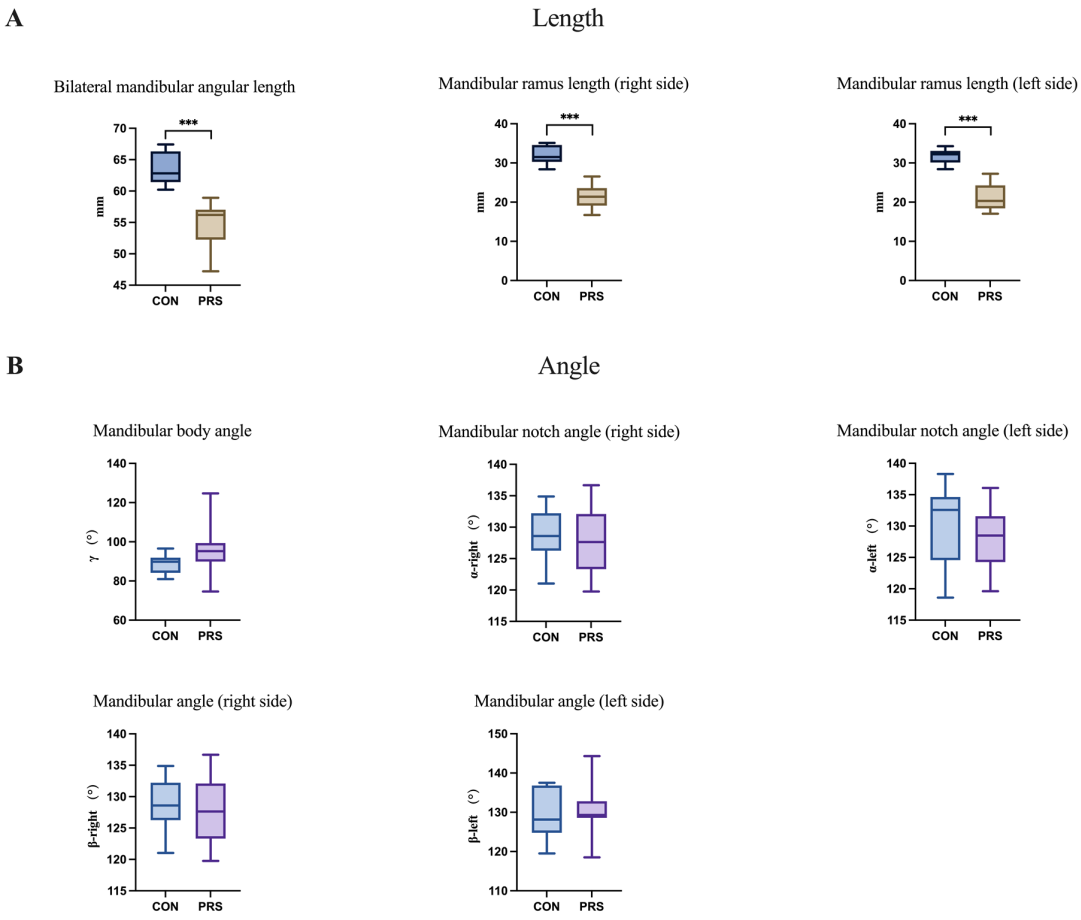

Figure 4. (A) Differences in mandibular length, including distance of bilateral mandibular angular and mandibular ramus (right side and left side) measurements between PRS and control groups following 3D reconstruction; **(B)** Differences in mandibular angle, including mandibular body, mandibular notch angle (right side and left side) and mandibular angle (right side, left side) measurements between PRS and control groups following 3D reconstruction. CON, control group; PRS, Pierre Robin Sequence group. $^{**}p < 0.01$ *vs.* CON group.

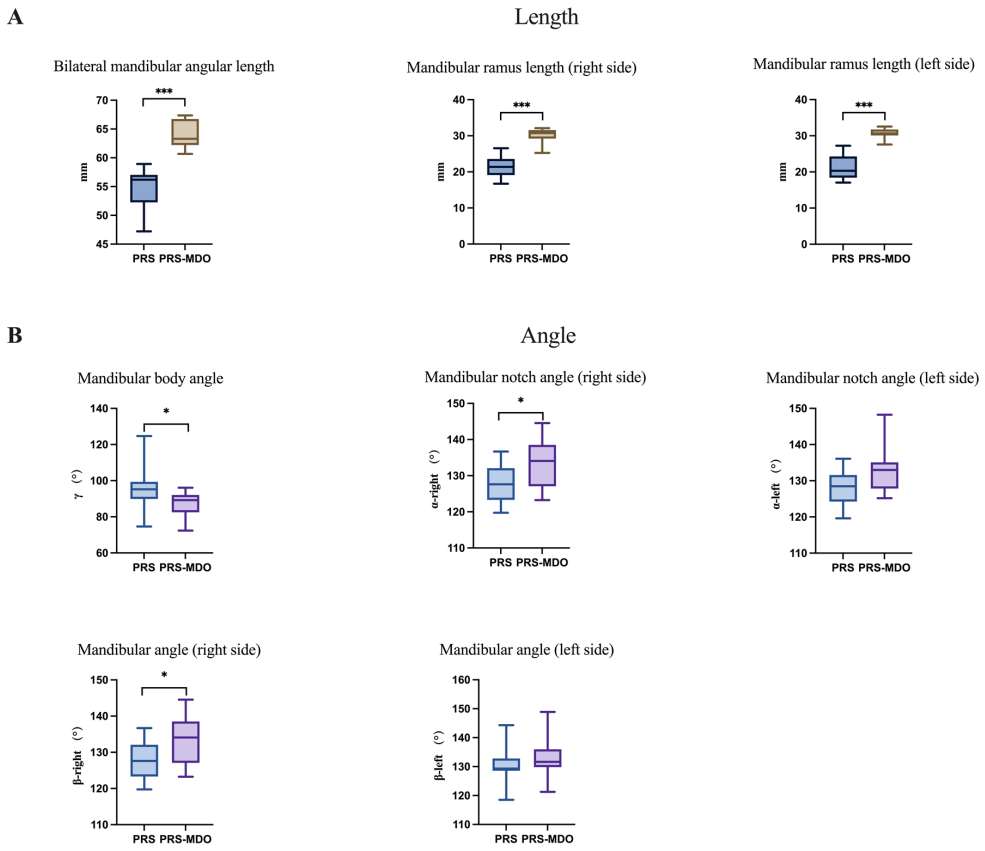

Figure 5. (A) Differences in mandibular length, including distance of bilateral mandibular angular and mandibular ramus (right side and left side) measurements between PRS and after MDO groups following 3D reconstruction. **(B)** Differences in mandibular angle, including mandibular body, mandibular notch angle (right side and left side) and mandibular angle (right side, left side) measurements between PRS and after MDO following 3D reconstruction. ***$p < 0.01$ *vs.* PRS group, *$p < 0.05$ *vs.* PRS group.

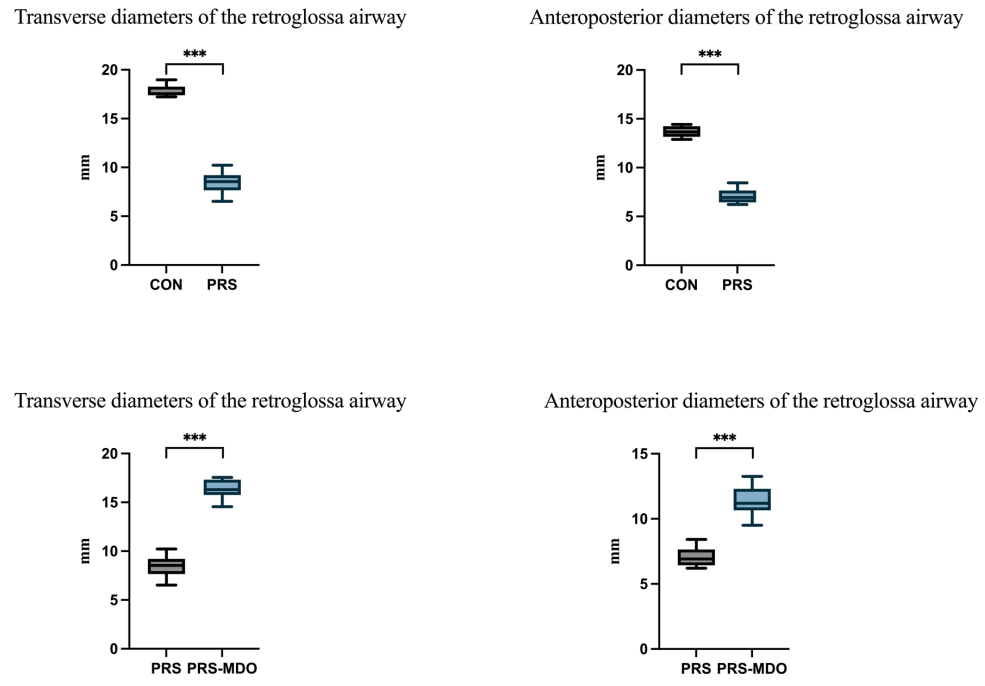

Figure 6. Differences in diameters of the retroglossal airway, including transverse and anteroposterior measurements among control, PRS and after MDO group following 3D reconstruction. Statistical significance is indicated by ***$p < 0.01$. PRS, Pierre Robin Sequence; MDO, Mandibular Distraction Osteogenesis.

that MDO effectively improves mandibular morphology (Figure 5A). Additionally, the angle of the mandibular ramus increased, while the angle of the mandibular body decreased compared to the PRS group ($p < 0.05$) (Figure 5B).

The transverse and anterior diameter of the posterior lingual airway, which are indicative of airway stenosis, were significantly smaller in the PRS group — almost half the size of the control group ($p < 0.01$). After MDO surgery, these diameters increased significantly ($p < 0.01$) (Figure 6).

3.4. Multiple linear regression analysis of mandibular and airway morphology with swallowing function

A stepwise regression analysis was conducted to examine the impact of various anatomical factors on the swallowing score. Based on differential analysis, five covariates with statistically significant differences were identified: distance between bilateral mandibular angular, mandibular ramus (right side), mandibular ramus (left side), transverse diameters of the retroglossal airway, and anteroposterior dimension of the retroglossal airway. The result was consistent with multivariate logistic regression models (Figure 7A) and SHAP algorithm (Figure 7B). To address potential multicollinearity, a correlation analysis was performed. A strong positive correlation was observed between the distance between bilateral mandibular angular and the mandibular ramus (left side) ($r = 0.864$, $p < 0.01$).

To minimize the impact of multicollinearity on the regression model, stepwise regression analysis was conducted in two approaches. First, using the distance between bilateral mandibular angular, after excluding the mandibular ramus (left side), stepwise regression analysis was performed with the distance between bilateral mandibular angular and the other covariates. The results indicated that the transverse diameters of the retroglossal airway (B = -0.246, t = -9.600, $p < 0.001$) and mandibular ramus (right side) (B = -0.088, t = -2.077, $p = 0.046$) were significant predictors of the swallowing score (dependent variable). Then using mandibular ramus (left side), after excluding distance between bilateral mandibular angular, stepwise regression analysis was performed with the mandibular ramus (left side) and the other covariates. The results showed that the mandibular ramus (left side) significantly influenced the swallowing score, with an unstandardized coefficient of B = -0.200 (t = -10.195, $p < 0.001$).

By including either distance between bilateral mandibular angular or the mandibular ramus (left side) in separate models, multicollinearity was reduced, and the relationship between the covariates and the dependent variable was clarified. These findings highlight the importance of careful selection and evaluation of covariates in regression modeling.

4. Discussion

PRS is a congenital condition characterized by mandibular hypoplasia, glossopteris, which often leads to respiratory distress, feeding difficulties, and various other complications, all of which significantly impact patient survival rates (*3*). PRS has an incidence of 1 in 8,500 to 1 in 30,000 live births. Mortality rates are significant, with an average of 16% across cases, but rising to 41.4% in severe forms. Reports indicate early infant mortality rates of 30% to 65%, driven by airway obstruction and feeding difficulties (*5,17,18*). Timely interventions, such as MDO, are crucial to improving

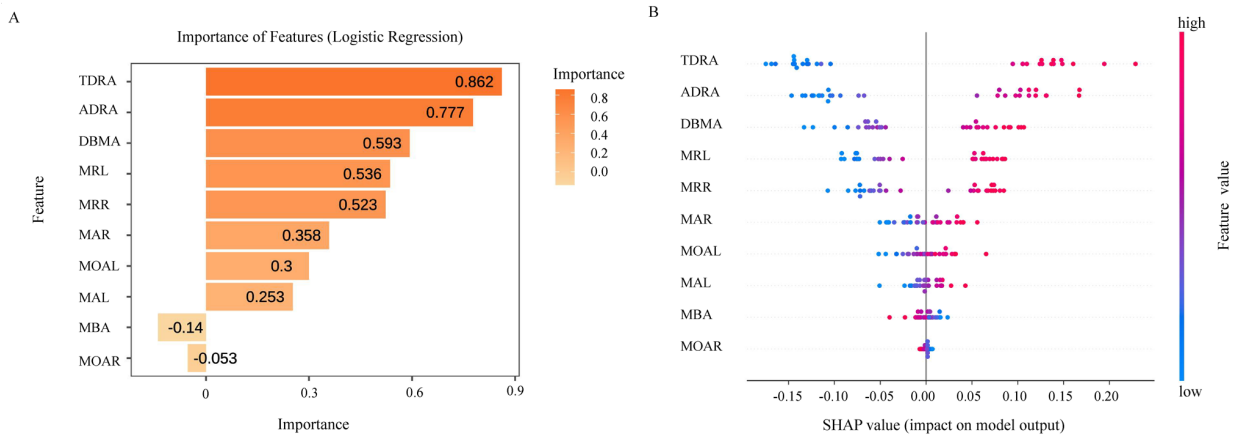

Figure 7. Feature importance evaluation using the logistic regression (LR) model and SHAP algorithm. (A) The Importance (coefficients of features) ranking of features in LR model; **(B)** SHAP honeycomb diagram of the LR model. Each point represents a feature value, and different colors represent the final influence of the feature on the LR model output results, where red represents a larger SHAP value and blue represents a smaller SHAP value. TDRA, Transverse diameters of the retroglossal airway; ADRA, Anteroposterior diameters of the retroglossal airway; DBMA, Distance between bilateral mandibular angular; MRL, Mandibular ramus (left side); MRR, Mandibular ramus (right side); MAR, Mandibular angle (right side); MOAL, Mandibular notch angle (left side); MAL, Mandibular angle (left side); MBA, Mandibular body angle; MOAR, Mandibular notch angle (right side).

outcomes.

The findings of this study confirm that MDO effectively increases mandibular length and adjusts the mandibular angle, bringing it closer to normal. Studies using 3D morphological analysis reveal that different types of PRS patients exhibit distinct mandibular characteristics compared to normal children. These differences may be associated with factors such as condylar rotation direction, rotation angle, and mandibular body elongation (*12*). Furthermore, research shows that MDO may affect the temporomandibular joint (TMJ). Following unilateral mandibular distraction, anatomical changes and mild degenerative alterations in the TMJ may occur, but these changes generally resolve over time, indicating that the short-term effects of MDO on the TMJ are largely reversible (*19*). Studies on adult patients with hemifacial microsomia have shown that MDO effectively reduces recurrence rates, as demonstrated by improvements in the ratio of ramus length, body length, and the distance from the chin point to the facial midline (*20,21*). These findings underscore the significant impact even minor variations in mandibular length and angle can have on mandibular development in PRS patients, highlighting the critical importance of precise preoperative planning, surgical strategy, and postoperative evaluation to achieve optimal therapeutic outcomes. Furthermore, the use of Mimics 3D reconstruction technology in this study enhances the accuracy of mandibular measurements (*22*). Scholars such as Chelsea L Reighard, have demonstrated that 3D printing technology allows for precise prediction of postoperative mandibular changes, facilitating surgical planning, improving outcomes, and reducing complications (*23*).

Mandibular morphology plays a critical role in swallowing function. As a key component of the oral system, the mandible directly influences essential oral functions like mastication and swallowing (*24*). Mandibular morphological features, such as the mandibular angle and ramus shape, are closely associated with masticatory muscle strength (*25*). In PRS patients, who typically present symptoms in the neonatal period before the establishment of dental arches, the position and shape of the mandible significantly impact the size of the oropharyngeal airway. For instance, mandibular retrognathia can reduce the size of the oropharyngeal airway and alter the hyoid bone's position, moving it upward and backward (*26,27*). This suggests that mandibular morphological abnormalities in PRS patients directly affect swallowing function. Our study results indicate that the lateral dimension of the retroglossal airway and the distance between bilateral mandibular angles are closely related to swallowing function.

In PRS patients, mandibular shortening and retrognathia commonly result in glossoptosis and upper airway narrowing, which subsequently impair swallowing function (*28*). Accurate evaluation of swallowing dysfunction in these patients is essential. For mild cases, intermittent oral feeding paired with perioral stimulation can improve swallowing (*28-31*). In severe cases where swallowing difficulties compromise growth and development, MDO surgery is necessary. This procedure effectively elongates the mandible, increases the oropharyngeal airway volume, and improves swallowing function (*32*). Treatment strategies for PRS patients should consider both mandibular morphology and swallowing needs to ensure the most suitable approach (*28*). This study underscores MDO as one of the most effective treatments, improving airway patency and alleviating respiratory and swallowing dysfunction by lengthening the mandible.

In summary, PRS is a congenital condition with a relatively low incidence but a high mortality rate. Early diagnosis and comprehensive treatment are essential for improving the survival rates of affected newborns. Treatment strategies should not only focus on alleviating airway obstruction but also address the impacts of swallowing dysfunction. Future research should aim to refine surgical indications for MDO, optimize preoperative planning, improve surgical techniques, and enhance postoperative care. Additionally, genetic testing plays a crucial role in personalized diagnosis and precision treatment (*33*). These advancements will help manage airway obstruction and swallowing issues more effectively, minimize secondary mandibular deformities, reduce surgical complications, and improve bone regeneration quality.

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Original Article

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Research hotspots and trends of the *SLC26A4* gene-related hearing loss from the perspective of knowledge graph

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SUMMARY: This article aims to identify research hotspots and trends in research on *SLC26A4* gene-related hearing loss through bibliometric and visual analyses, providing a reference and direction for future research. Publications on *SLC26A4* gene research in hearing loss from 1994 to 2023 were retrieved from the Web of Science Core Collection database. Bibliometric analysis was conducted using the Bibliometrix 4.0.0 R package, CiteSpace 6.2.R6 software, and VOSviewer 1.6.20. The analysis encompassed journals, authors, keywords, institutions, countries, and references. Based on the analysis results, network maps were generated to evaluate collaborations among authors, countries, institutions, keyword co-occurrences, and co-citation references. This study identified 1,308 publications from 62 countries. Annual publication numbers have increased with fluctuations, showing rapid growth since 2011. The USA emerged as the leading contributor in this field based on scientific production, citations, and cooperation networks. *International Journal of Pediatric Otorhinolaryngology* had the highest number of publications, while *Laryngoscope* was the most cited journal. Harvard University was the most productive institution. Key researchers included Dai Pu, Griffith Andrew J., and Usami Shin-Ichi. There have been active collaborations between countries, authors, and institutions. The primary research topics focused on genotype-phenotype correlations, genetic screening, diagnostic advancements, and exploration of pathogenic mechanisms. Research on *SLC26A4* gene-related hearing loss has notably increased since 2011, with ongoing clinical investigations and basic research efforts. Future studies may further explore disease mechanisms and potential therapeutic interventions related to the *SLC26A4* gene.

Keywords: *SLC26A4* gene, hearing loss, hotspots, research trends, visualization analysis

1. Introduction

Hearing loss (HL) is a prevalent sensory defect globally, with both syndromic and non-syndromic HL commonly inherited and linked to over 250 genes (*1*). *SLC26A4* gene mutation is one of the leading causes of hereditary HL worldwide (*2*). *SLC26A4* (solute carrier family 26, member 4), also known as PDS, is the causative gene of Pendred syndrome and autosomal recessive nonsyndromic deafness 4 (DFNB4) (*3,4*). Both Pendred syndrome and DFNB4 are associated with enlargement of the vestibular aqueduct and HL. *SLC26A4* encodes pendrin, which is expressed in the inner ear, thyroid gland, and kidney (*3,5,6*). Pendrin functions as an anion exchanger for Cl^-/I^- and Cl^-/HCO_3^- across apical plasma membranes of epithelial cells (*7,8*).

Researchers worldwide have conducted numerous clinical and basic studies on *SLC26A4* gene-related HL (*7*). However, there remains a gap in comprehensively exploring the current research status and future trends of *SLC26A4* in HL through the lens of a scientific knowledge graph. Research in this field has mainly explored specific topics without thoroughly examining research evolution from an international and comprehensive standpoint and without using knowledge graph tools for multi-angle visual research. Moreover, there is a lack of in-depth investigation into research hotspots, emerging frontiers, and evolving topic trends.

This study utilized bibliometric tools including Bibliometrix, CiteSpace, and VOSviewer to analyze and visualize a scientific knowledge map of the *SLC26A4* gene in HL research. International literature published between 1994 and 2023 was retrieved from the Web of Science Core Collection database. In addition, this study analyzed key information in this field, including journals, countries, institutions, authors, keywords, and references. The goal of this study was to assess research hotspots and trends in *SLC26A4* gene research in HL, providing a reference and direction for follow-up research.

2. Materials and Methods

2.1. Data collection

We conducted a comprehensive search of the science citation index expanded (SCI-E) database in the Web of Science Core Collection to obtain all relevant publications. A search query was conducted using the following formula: (TS = "SLC26A4" OR TS = "SLC26A4 gene" OR TS = "Pendred syndrome" OR TS = "Pendred syndrome gene" OR TS = "PDS" OR TS = "PDS gene" OR TS = "large vestibular aqueduct" OR TS = "large vestibular aqueduct syndrome" OR TS = "enlarged vestibular aqueduct" OR TS = "large vestibular canal" OR TS = "large vestibular canal malformation") AND (TS = "hearing" OR TS = "auditory" OR TS = "audiological" OR TS = "audiology" OR TS = "hearing loss" OR TS = "deafness" OR TS = "hearing impairment" OR TS = "hearing disorder" OR TS = "hearing defect" OR TS = "hearing handicap" OR TS = "hearing difficulty").

2.2. Screening criteria

The retrieval period spanned from January 1994 to December 2023, and the search language was limited to English. This search strategy yielded 1,578 records collected on March 25, 2024. Subsequently, we filtered the results by document type to include only articles. Reviews, editorials, letters to the editor, retracted publications, book chapters, meeting abstracts, proceedings, and papers with early access were excluded. After applying the inclusion criteria, 1,308 publications were ultimately included in this study. The study design is illustrated in Figure 1.

2.3. Bibliometric analysis

Bibliometric analysis and visualization were conducted using Bibliometrix (version 4.0.0), CiteSpace (version 6.2.R6), and VOSviewer (version 1.6.20). Bibliometrix is an R package that provides scientific mapping workflow from data collection to data visualization. CiteSpace is a Java-based software made especially for bibliometric network analysis and visualization. VOSviewer is software used to construct and visualize bibliometric networks based on citation, co-citation, co-authorship, or term co-occurrence relations. Multiple points extracted from the dataset were used to conduct the bibliometric analysis, including journals, countries, institutions, authors, keywords, cited references, publication years, titles, abstracts, document types, number of citations, and time of citations. Additionally, the h-index and g-index, two crucial bibliometric metrics for exploring scientific production and impact, were gathered (*9,10*). The data were then imported into the bibliometric tools; Bibliometrix, CiteSpace, and VOSviewer for analysis. Network maps generated by these tools include collaboration networks, co-occurrence, and co-citation (*11,12*).

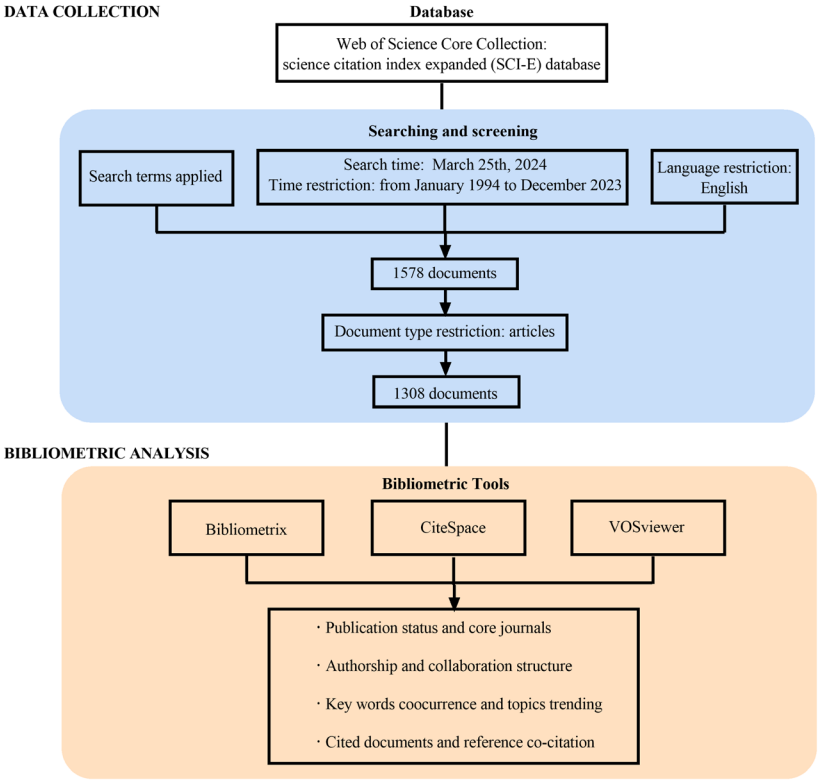

Figure 1. Data collection and bibliometric analysis of this study.

2.4. Statistical analysis

Bibliometrix analysis included key information from the dataset such as annual publications, journal production, publication citations, countries' production, institutions' production, authors' production, cited references, core journals based on Bradford's law, country collaboration maps, author productivity according to Lotka's law, and author's production over time. CiteSpace analysis included collaboration networks and the centrality value of countries, institutions, and authors; keywords with the strongest citation bursts; and the co-citation reference network. VOSviewer was used to generate a co-occurrence network and keyword clusters.

3. Results

3.1. Annual global publication volume

Between 1994 and 2023, 1,308 articles related to *SLC26A4* gene-related HL were published worldwide (Table 1). The annual publication trends show a fluctuating increase, as shown in Figure 2. The number of papers published in 1999, 2001, 2011, 2013, 2016, and 2017 showed rapidly increasing trends. The highest number of publications (NP) (80) was in 2017. The overall increase in publications can be roughly categorized into three stages based on yearly scientific production: the first stage (1994–2010), showing slow and stable development; the second stage (2011–2017), demonstrating continuous and rapid development; and the third stage (2018-2023), indicating a fluctuating trend.

Table 1. Main information from the dataset used in this study after manual screening

Main Information	Details
Timespan	1994-2023
Documents	1,308
Keywords	2,116
Reference	21,197
Countries	62
Institutions	1,515
Authors	5,792
Journals	337
Language	English

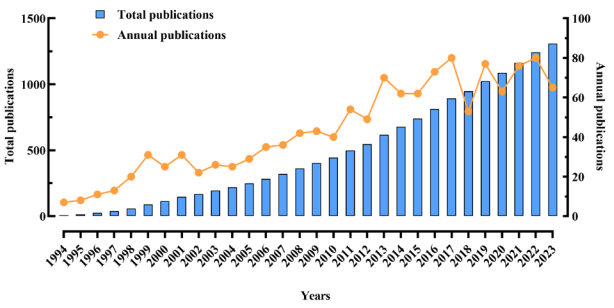

Figure 2. Total publications and annual publication distribution of research on *SLC26A4* gene-related hearing loss from 1994 to 2023. The blue bars represent the accumulation of articles, while the orange line demonstrates the annual scientific production.

3.2. Core journals, authorship, and collaboration

3.2.1. Core journals based on Bradford's law

Bradford's law is an essential and fundamental principle in bibliometric analysis that describes the distribution of scientific publications in journals (*13,14*). The core journals were analyzed using Bradford's law (Supplemental Figure S1, *https://www.irdrjournal.com/action/getSupplementalData.php?ID=236*). The 1,308 articles published in 337 journals, and the top 10 most productive journals are listed in Table 2. These top journals published up to 32.34% (423/1,308) articles and represented only 2.97% (10/337) of all journals. The top 3 journals were the *International Journal of Pediatric Otorhinolaryngology*, *Otology & Neurotology*, and *Acta Oto-Laryngologica*, with 115, 81, and 48 published articles, respectively. Table 2 displays the NP, total citations (TC), h-index, g-index, and core journal categories. *Laryngoscope* ranked first in TC (1792), h-index (24), and g-index (42), while *Ear and Hearing* ranked first in Journal Impact Factor (JIF) quartile (Q1).

3.2.2. Scientific production and cooperation of countries

A total of 62 countries and regions were involved in the publication of research. As shown in Figure 3A, the countries and regions with the top three scientific production in this field are the United States, China, and Japan. The United States ranked first with 329 publications and led in terms of TC (13,548) (Supplemental Table S1, *https://www.irdrjournal.com/action/getSupplementalData.php?ID=236*). Figure 3B displays the average article citation counts for leading scientific nations, with France, the United Kingdom, the USA, and Australia ranking highest.

Cooperation between countries generated by CiteSpace is shown in Figure 3c, and a country collaboration map created *via* Bibliometrix is presented in Supplemental Figure S2 (*https://www.irdrjournal.com/action/getSupplementalData.php?ID=236*). According to the cooperation network, the more visible significant collaborations are the connections between the USA and European countries, such as Italy and Germany, and Asian countries, such as China and Japan. A close cooperation sub-network was observed among European countries.

International cooperation is correlated with the ratio of multiple-country production (MCP) to single-country production (SCP) and can be evaluated by the percentage of MCP in the total NP. The findings of this dataset (Figure 3A; Supplemental Table S1, *https://www.irdrjournal.*

Table 2. Top 10 most productive journals sorted by number of publications

Journals	NP	TC	h-index	g-index	JIF quartile and JCR categories
1. International Journal of Pediatric Otorhinolaryngology	115	1,168	20	34	Q3, Otorhinolaryngology Q4, Pediatrics
2. Otology & Neurotology	81	1,624	24	37	Q3, Otorhinolaryngology Q4, Clinical Neurology
3. Acta Oto-Laryngologica	48	645	15	23	Q4, Otorhinolaryngology
4. Laryngoscope	44	1,792	24	42	Q2, Otorhinolaryngology Q3, Medical, research, and experimental
5. Plos One	40	1,252	21	34	Q2, Multidisciplinary sciences
6. European Archives of Oto-Rhino-Laryngology	25	251	11	15	Q2, Otorhinolaryngology
7. Ear and Hearing	19	251	10	15	Q1, Otorhinolaryngology, Q1,Audiology, and speech-language pathology
8. Journal of Laryngology and Otology	19	221	10	14	Q3, Otorhinolaryngology
9. Annals of Otology, Rhinology, and Laryngology	16	670	13	16	Q4, Otorhinolaryngology
10. Clinical Genetics	16	618	13	16	Q2, Genetics and heredity

NP: number of publications; TC: total citations; JIF: Journal Impact Factor; JCR: Journal Citation Reports.

com/action/getSupplementalData.php?ID=236) show that the countries with the highest cooperation rates are Belgium (43.33%), Germany (35.00%), and the United Kingdom (33.33%). Centrality measures the likelihood of the shortest path passing through a node and reflects its importance in the network. Countries with the top five centralities were the United States (0.65), Germany (0.25), the United Kingdom (0.22), France (0.17), and Italy (0.11), indicating that these five countries also have significant roles in this field (Figure 3C; Supplemental Table S1, *https://www.irdrjournal.com/action/getSupplementalData.php?ID=236*).

3.2.3. Scientific production and cooperation of institutions

The top 10 contributing institutions were located in the USA, South Korea, China, and Japan (Supplemental Table S2, *https://www.irdrjournal.com/action/getSupplementalData.php?ID=236*). Regarding the NP, Harvard University and Harvard Medical School ranked the highest. With regard to the total global citations, the National Institutes of Health (NIH), USA, and the NIH National Institute on Deafness and Other Communication Disorders (NIDCD) take the lead.

The cooperation network of institutions (Figure 4A) demonstrated close collaboration and connections among research institutions focusing on *SLC26A4* gene research in HL. Affiliated institutions from various nations are dispersed and intimately connected across the map, particularly because institutions within the same region collaborate more frequently.

3.2.4. Scientific production and cooperation of authors

Lotka's law is a bibliometric and information science principle that characterizes the productivity distribution of authors on a specific topic (*15*). The authors' productivity using Lotka's law is shown in Supplemental Figure S3 (*https://www.irdrjournal.com/action/getSupplementalData.php?ID=236*). The top three productive authors in terms of NP, TC, and h-index were Dai Pu from China (32, 791, and 15, respectively), Griffith Andrew J. from the USA (29, 1,409, and 20, respectively), and Usami Shin-Ichi from Japan (29, 1,204, and 18, respectively) (Supplemental Table S3, *https://www.irdrjournal.com/action/getSupplementalData.php?ID=236*). According to scientific production over time, the top 10 authors (Supplemental Figure S4, *https://www.irdrjournal.com/action/getSupplementalData.php?ID=236*) are from China, USA, Japan, South Korea, and Taiwan. Griffith Andrew J., Smith Richard J. H., and Usami Shinichi have been active in this field since 1996, 1997, and 1999, respectively. Other researchers joined and contributed development in 2005. By 2008, nine of the top 10 authors were dedicated to this field.

Figure 4B shows the collaboration network map of the authors. Numerous closely connected domestic and international clusters exist within the authors' collaborative networks. The authors were divided into several clusters that exhibited close internal interactions. For instance, Griffith Andrew J. from NIH engaged with research teams in the USA, Japan, South Korea, and Taiwan. Dai Pu, Yuan Yongyi, and Huang Shasha,

Figure 3. Countries' scientific productions and collaboration relationships. (A) Scientific production and international collaboration by countries. MCP represents multiple-country production, and SCP represents single-country production; **(B)** Average article citations by countries; **(C)** Collaboration network of countries. Each node's size represents the citations of each country. Node colors closer to the center indicate earlier publication years, while exterior colors indicate more recent publication years. The purple circle represents the node's centrality ≥ 0.1, and the circle's size is proportional to the centrality value.

from the same institution, were also very close to the collaboration map.

3.3. Research on trending topics

3.3.1. Trending topics derived from keyword co-occurrence and clustering

The most frequent keyword visualization is presented in a word cloud map (Supplemental Figure S5, *https://www.irdrjournal.com/action/getSupplementalData.php?ID=236*). A keyword co-occurrence network was generated using VOSviewer to explore keyword co-occurrence and clustering further. A total of 193 keywords, each with a minimum occurrence of 20, were used to generate the network plot (Figure 5). Using VOSviewer, three clusters — green, blue, and red (Figure 5A) — representing various research topics were identified. Additionally, the average citations of these keywords were generated and displayed in Figure 5B.

3.3.2 Trending topics bursts based on keywords

The top 25 keywords with the strongest citation bursts in research are demonstrated in Supplemental Figure S6, *https://www.irdrjournal.com/action/getSupplementalData.php?ID=236*. The blue line represents the period from 1994 to 2023, while the red line indicates the interval between the burst keywords. The keywords with the highest burst strength over the past 30 years are "large vestibular aqueduct" (13.94), "sensorineural HL" (13.76), and "spectrum" (11.01). Other burst keywords in the last 5 years contain "genotype" (2019–2023), "inner ear malformation" (2019–2023), and "guidelines" (2020–2023). From 1994 to 2003, trending topics included "large vestibular

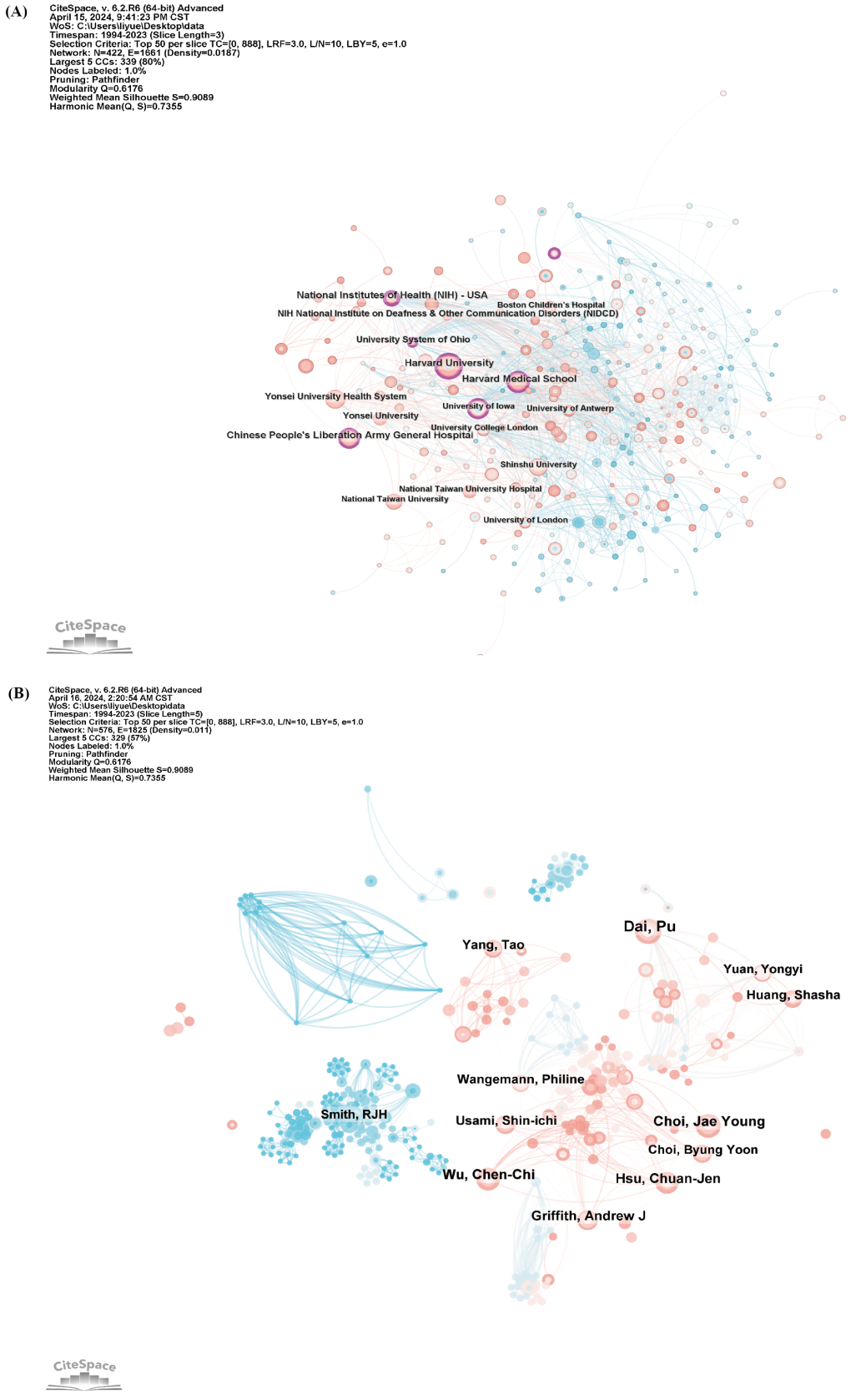

Figure 4. Collaboration network. (A) Institutions' collaboration network. **(B)** Authors' collaboration network. Each node's size represents the citations for each institution or author. Node colors closer to the center indicate earlier publication years, while exterior colors indicate more recent publication years. The purple circle represents the node's centrality ≥ 0.1, and the circle's size is proportional to the centrality value.

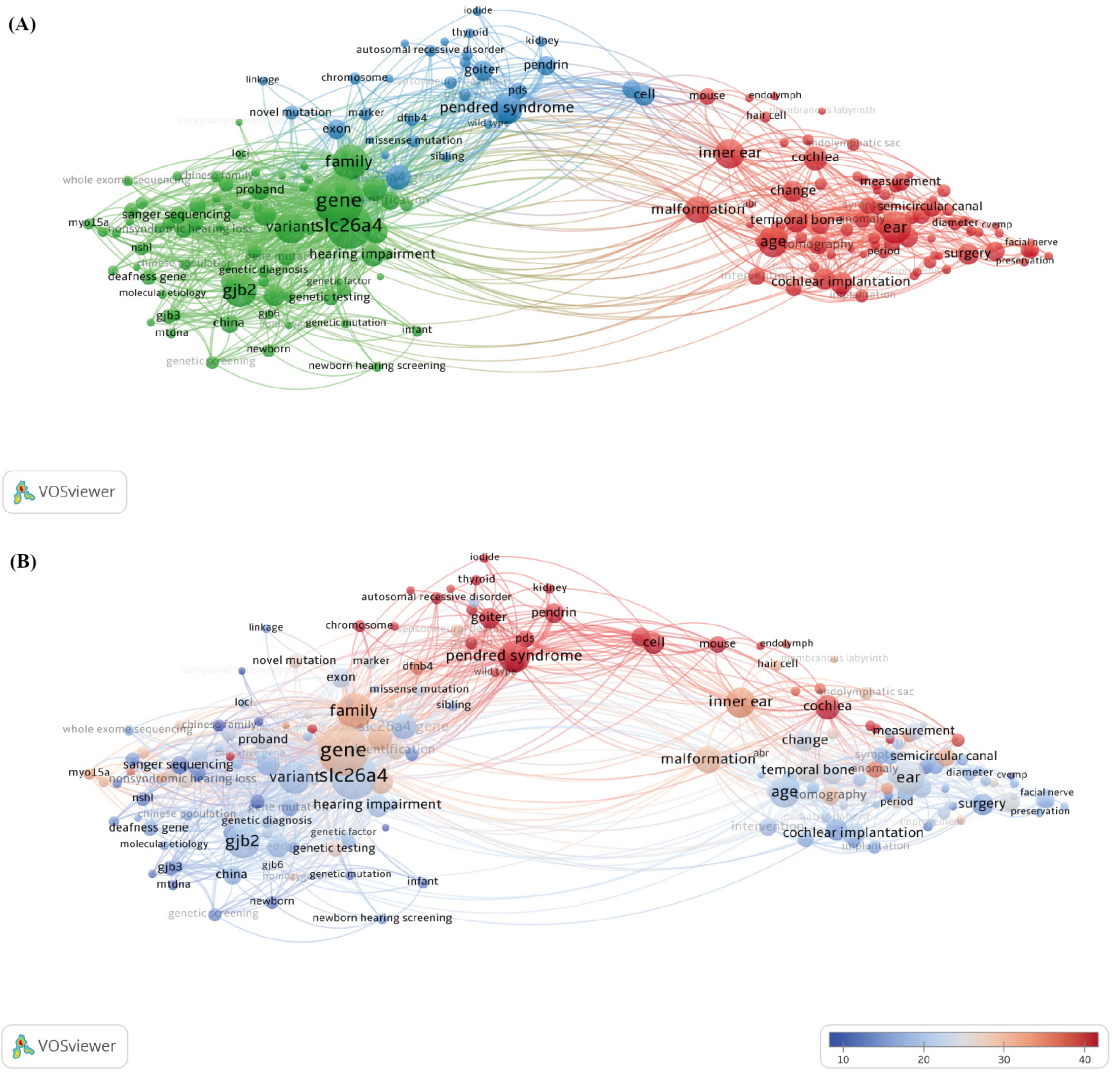

Figure 5. Co-occurrence network of keywords. (A) Clusters; **(B)** Keywords' average citations. Minimum number of occurrences for a term is set at 20, resulting in 193 keywords meeting the threshold. The relative distance between two nodes generally indicates the intensity of their association, and the size of nodes is proportional to the frequency of keywords. Keywords with larger circles indicate research hotspots.

aqueduct", "sensorineural HL", "linkage", and "endolymphatic sac", focusing more on phenotype and tentative anatomical explorations of *SLC26A4* gene-related HL.

3.4. Hotspots derived from publication and reference co-citation

3.4.1. Principal publications

Based on each publication's citation frequency, Bibliometrix was used to determine which articles were the most cited globally. Supplemental Table S4 (*https://www.irdrjournal.com/action/getSupplementalData.php?ID=236*) lists the relevant data for these influential publications. The most globally cited publication was published by Everett *et al*. in 1997 (*3*), focusing on identifying *SLC26A4* gene mutations associated with Pendred syndrome. Three other highly globally cited publications by Scott *et al.* (*16*), Royaux *et al.* (*17*), and Everett *et al.* (*5*) in 1999, 2001, and 2001, respectively, launched new areas of investigation into *SLC26A4* and pendrin proteins, exploring inner ear pathology associated with *SLC26A4* gene mutations through cell experiments and animal models.

3.4.2 Hotspots' evolution trends derived from cited references

Cited references form the scientific foundation of a specific research area (*18*). Supplemental Table S5 (*https://www.irdrjournal.com/action/getSupplementalData.php?ID=236*) presents the top 10 references in the field with the highest citations. The top three cited references are Everett *et al.* (1997) (*3*), Valvassori and Clemis (1978) (*19*), and Park *et al.* (2003) (*2*). These references primarily discuss the mutation frequencies and spectrum of the *SLC26A4*

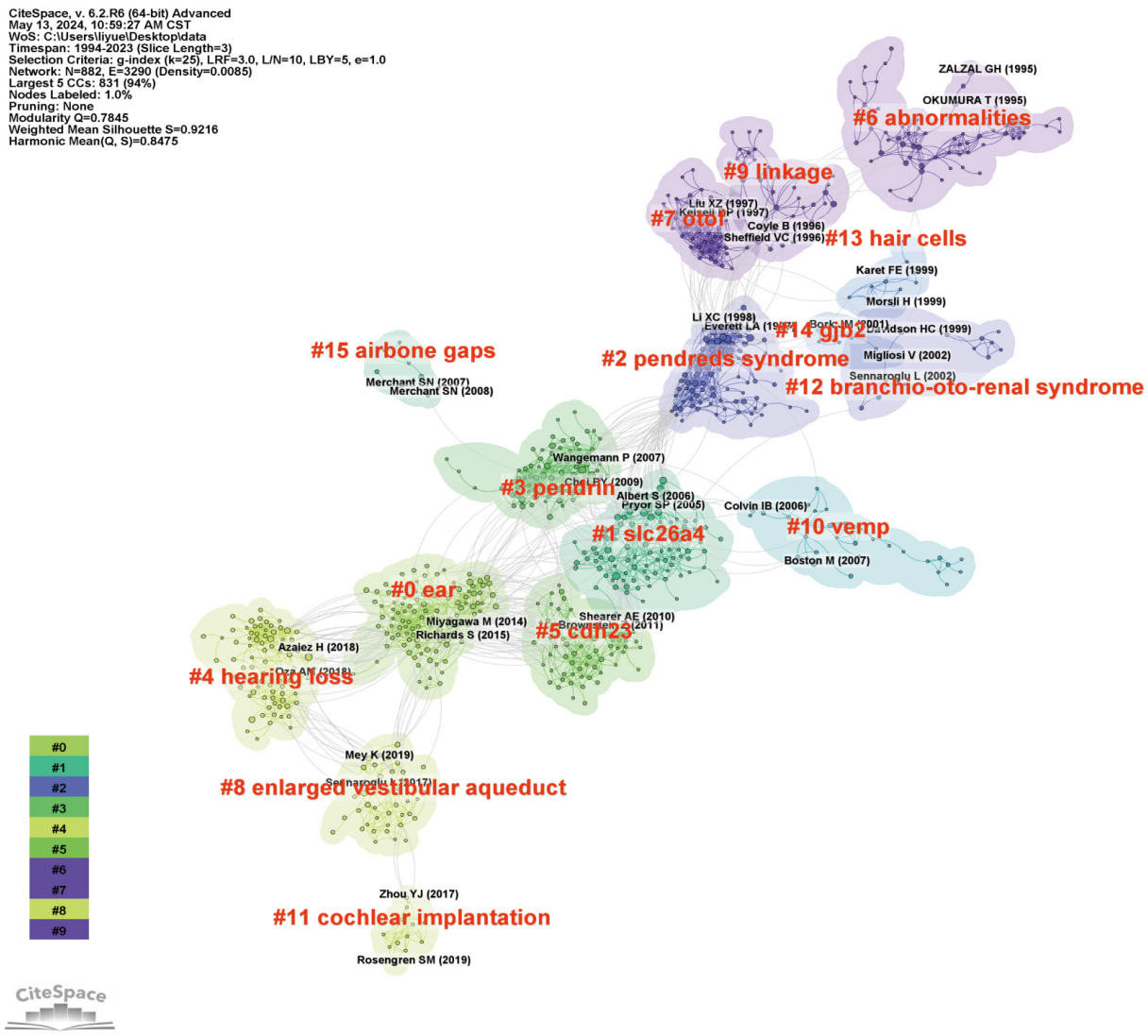

Figure 6. Co-citation reference network with cluster visualization. The size of each node represents the number of citations for each reference.

gene and genotype-phenotype correlations.

To investigate the theoretical underpinnings of the research, we used CiteSpace to create a co-citation network of references. The largest 15 clusters are summarized in Figure 6. Node significance is encapsulated by citation metrics such as counts and bursts, as well as network metrics like betweenness and degree centralities.

The largest cluster (#0) included 110 members and had a silhouette value of 0.853. The most cited members of this cluster are Richards *et al.* (2015) (*20*) and Miyagawa *et al.* (2014) (*21*). The second-largest cluster (#1) had 102 members with a silhouette value of 0.885. The most cited members of this cluster are Pryor *et al.* (2005) (*22*) and Albert *et al.* (2006) (*23*). The third-largest cluster (#2) had 88 members and a silhouette value of 0.915. The most cited members of this cluster are Everett *et al.* (1997) (*3*) and Li *et al.* (1998) (*4*).

4. Discussion

This study utilized bibliometric and visual analysis tools to conduct a comprehensive analysis of research literature on *SLC26A4* gene-related HL within the Web of Science database. A total of 1,308 publications by 5,792 authors from 337 journals were obtained, providing an overview of the research landscape, frontiers, hotspots, and trends. From 1994 to 2023, the annual publications increased with fluctuations in three stages. A rapid increase was observed between 2011 and 2017.

Core journal analysis revealed that *International Journal of Pediatric Otorhinolaryngology* published the most papers. Noteworthy contributions have been made by *Laryngoscope, Ear and Hearing,* and other core journals in terms of TC and JIF. Regarding the TC of publications, Everett *et al.*'s (*3*) publication in *Nature Genetics* was the most frequently cited, with 888 citations.

Approximately 5% of the global population suffers from HL issues, with 3-5% of these cases attributed to mutations in the *SLC26A4* gene, which vary by ethnicity (*24-26*). Studies on nonsyndromic deafness reported biallelic mutations in the *SLC26A4* gene were present in 2% to 3.5% of Caucasian patients (*27-29*), while these mutations frequencies were higher in East Asian patients, ranging from 5.5% to 12.6% (*21,30-32*). The c.716T>A mutation was the most commonly observed in Turkey and Pakistan (*2*), whereas c.1238A>G and c.919-2A>G occurred more frequently as mutations in Iran (*33*,*34*) and c.919-2A>G and c.1334C>T were more commonly found in East Asia (*21,35,36*). Given the wide mutation spectrum of the *SLC26A4* gene, its related hearing loss has attracted global attention, involving researchers from 62 countries. The USA (25.15%), China (23.85%), and Japan (7.34%) ranked among the top three contributing countries, accounting for 56.35% of the total publications. The USA had the most influential impact in this field based on scientific output, average article citations, and cooperation networks. Notably, publications from France, the United Kingdom, and Australia also received high-average article citations, indicating high-quality research. The USA, Germany, the United Kingdom, France, and Italy have had high centrality in recent decades, which indicates that related research in this field from these countries is taking a leading place. Additionally, we found that the rate of international cooperation is generally higher in European countries than in non-European countries and higher in developed countries than developing ones. Contributions in this field from various countries could differ for various reasons, including differences in the ethnicity of patients with HL, diverse cultural backgrounds, financial factors, and medical levels.

Universities and public research institutes are the prominent affiliated institutions involved in research. Research institutions such as the NIH-USA, University of Iowa, NIDCD, University System of Ohio, Harvard University, Harvard Medical School, and the Chinese People's Liberation Army General Hospital contributed to high centrality. Close links were observed in the institutions' collaboration networks, suggesting that international cooperation among institutions is extensive.

Dai (China), Griffith (USA), and Usami (Japan) were the three most productive authors in the field. Griffith, Usami, Smith, and Wangemann each received over 1,000 citations in their publications. The top 15 productive authors were from China, the USA, Japan, Korea, and Taiwan. Compared to countries' collaboration networks and institutions' collaboration networks, we found that authors' collaboration networks showed fewer links and collaborative relationships, indicating potential for strengthening.

Regarding research hotspots, reference co-citation networks reflect the fact that researchers are mainly focused on clinical diagnosis, treatment, intervention, and laboratory research. "The large vestibular aqueduct syndrome" published by Valvassori (1978), is considered a classic reference in this field, analyzing radiographic observations of patients (*19*). Burst keywords reflect emerging trends and research frontiers. Frequent keyword visualizations generated by VOSviewer classify the green, blue, and red clusters into three categories: clinical audiological and genetic diagnosis, genotype-phenotype linkage and mechanical investigation, and clinical treatment. Certain keywords with low frequency (*e.g.*, "iodide", "thyroid", "kidney", "endolymph") received more citations. This finding might indicate that although these topics have received widespread attention from researchers, the number of relevant studies still needs to be improved. The more regularly cited keywords among the more frequently occurring group were "Pendred syndrome", "cochlea", "cell", and "mouse". These keywords are related to clinical symptoms and pathogenic mechanisms. In the last 10 years, research on genotype-phenotype correlations, genetic screening, and diagnosis has progressed rapidly (*7,21,37-39*). Building on this progress, our research group has also focused on *SLC26A4* gene-related hearing loss, including mutation analysis in Chinese families, the investigation of genotype-phenotype characteristics and the prediction models of hearing loss trajectory in children (*40-42*). We identified novel compound heterozygous mutations in *SLC26A4*, as well as mutations in related genes such as *FOXI1* and *KCNJ10* in infants with a single-allele *SLC26A4* mutation (*43,44*).

Around 1999, researchers started to expand the field to include "*PDS* gene", "goiter", "congenital deafness", "molecular analysis", "identification", "sulfate transporter", and "expression", which is more related to mechanism exploration and protein function. Since 1999, basic studies on animal and cell models have proliferated to explore its protein function and pathogenic mechanism (*5,6,16,17,45*). Genetic studies on patients with Pendred syndrome and mouse models have provided insights into the *SLC26A4* gene. However, the exchange mechanism of the *SLC24A4* coding protein, pendrin, remains unknown. With the development of experimental technologies, in 2023, single particle cryo-electron microscopy and anion exchange assays were used to explore the structure and function of pendrin, providing a structural basis for understanding pendrin and *SLC26A4* variants (*46*). A new era of precision gene therapy has emerged (*47*), with recent reports in 2024 of gene therapy for the treatment of children with *OTOF* gene-related HL (*48*). Moreover, several basic research articles have also investigated adeno-associated virus (AAV) gene replacement therapy for recessive hereditary hearing loss involving different genes (*49,50*). Among the datasets we analyzed, we found that there were also explorations into gene therapy with a recombinant adeno-associated virus (rAAV) to transfect *SLC26A4* cDNA in mouse models of *SLC26A4* gene-related HL (*51*), and

another therapy of rescuing *SLC26A4* gene-related HL with *DNAJC14* overexpression *via* Japanese encephalitis virus (JEV) activation (*52*), in mice. In addition, the CRISPR/Cas9-mediated exon skipping strategy (*53*) and the antisense oligonucleotides (ASOs) strategy (*54*) have also been reported to be utilized in mouse models of *SLC26A4* gene-related HL, indicating the feasibility of related therapy and providing a frontier research topic in this field, with potential challenges and opportunities.

There are some limitations to this research: *i*) The literature database we utilized was only the Web of Science, which might have resulted in incomplete literature retrieval; *ii*) Analysis using bibliometric tools such as Bibliometrix, CiteSpace, and VOSviewer might be inconsistent with experts' viewpoints in this field; and *iii*) There might be a selection bias in the publications analyzed.

5. Conclusion

Over the past 30 years, the number of annual publications has generally increased. Researchers from the USA have been the most influential and productive in *SLC6A4* gene research related to HL. Researchers from European and East Asian countries have also contributed significantly to the literature. Genotype-phenotype correlations, as well as genetic screening and diagnosis, are hot spots, alongside basic research on *SLC26A4* gene-related pathogenic mechanisms that have received continuous attention. Future research should focus on gaining deep insights into *SLC26A4* disease-associated mechanisms and therapeutic discoveries.

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

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Original Article

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miR-141-3p-loaded extracellular vesicles ameliorate intrahepatic bile duct stone disease by decreasing MUC5AC expression *via* the MAPK pathway

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SUMMARY: Intrahepatic bile duct stone disease has a high morbidity in China, with a high rate of additional surgery, a high rate of cancer development, and a high disease burden. Activation of the MAPK pathway leading to up-regulation of MUC5AC expression is an important factor in the formation of intrahepatic bile duct stones. Exosomes or extracellular vesicles (EVs) can be used as therapeutic vectors to encapsulate and carry drugs into diseased cells to achieve a therapeutic effect. The current study alleviated intrahepatic bile duct stone disease by preparing EVs carrying miR-141-3p. First, the researchers loaded mesenchymal stem cell (ESC)-derived EVs with miR-141-3p (miR-141-3p-EVs) and verified the phenotypes and characteristics of miR-141-3p-EVs. miR-141-3p-EVs successfully reduced the inflammatory level of human biliary epithelial cells (HIBEC) and lowered, *via* the MAPK pathway, MUC5AC expression. In an experiment involving an animal model of intrahepatic bile duct stones, miR-141-3p-EVs effectively alleviated stone formation, and the intrinsic mechanism was associated with the decreased level of MAPK pathway expression. In conclusion, results suggested that the EV-based strategy of miR-141-3p delivery to intrahepatic bile duct epithelial cells has value and provides a new approach for the treatment of intrahepatic biliary stone disease.

Keywords: hepatolithiasis, extracellular vesicles, miR-141-3p, MAPK, MUC5AC

1. Introduction

Intrahepatic bile duct stones (hepatolithiasis, or HL) occur above the confluence of the left and right hepatic ducts and may simultaneously be associated with extrahepatic bile duct stones (*1*). The prevalence of HL accounts for approximately 0.6% to 21.2% of primary cholelithiasis cases (*2*). HL is characterized by a prolonged and recurrent course that is difficult to cure. In the late stages of the disease, complications such as biliary cirrhosis, portal hypertension, and diffuse hepatic parenchymal destruction may occur, with a high risk of malignant transformation into intrahepatic cholangiocarcinoma (ICC), an extremely malignant cancer. Current research suggests that in China, HL is a significant risk factor for ICC, with a relative risk of 5.765. Globally, approximately 2% to 10% of HL cases may develop into ICC (*3-5*). Currently, the primary treatment for HL relies on surgical intervention. However, the postoperative residual stone rate is high, with a frequent recurrence of stones, leading to a reoperation rate ranging from 37.1% to 74.4%. Additionally, surgical procedures often result in severe complications, contributing to high morbidity and mortality rates. These factors present significant challenges in the clinical management of HL (*6-10*).

Bacteria and the infections they cause are significant contributors to the development of intrahepatic bile duct stones. Clinical studies have found that the bile of HL patients contains a large number of Gram-negative bacteria. Lipopolysaccharides (LPSes), which are one of their metabolic products, can stimulate biliary epithelial cells to activate cytokines and inflammatory mediators. This process leads to chronic proliferative inflammatory changes in the intrahepatic bile ducts, accompanied by the hypersecretion of mucin MUC5AC. This pathological change is considered a primary characteristic of HL (*11,12*). Previous studies have reported that miR-141-3p can negatively regulate the EGFR/ERK pathway, thereby inhibiting the progression of inflammation or tumors (*13-15*). Since LPS-induced overexpression of MUC5AC plays a critical role in the formation of intrahepatic bile duct stones, miR-141-3p may serve as a novel target for the prevention and treatment of this condition (*16*).

Small extracellular vesicles (sEVs) are bilayer membrane-like vesicles capable of carrying proteins, lncRNAs, miRNAs, and lipid components. The

protective nature of these vesicles prevents the degradation of these small molecules in the extracellular environment, allowing them to be taken up by recipient cells. Once inside the recipient cells, these molecules can regulate gene expression and protein synthesis, playing a crucial role in intercellular communication, surface modification, and other cellular functions (*17-20*). Exosomes derived from mesenchymal stem cells (dMSC-sEVs) are characterized by their safety and low level of immunogenicity, enabling the delivery of various small molecule drugs, chemotherapeutic agents, and RNA fragments. These exosomes have been utilized in several clinical trials and have yielded promising outcomes (*21*). Exosomes that enter the bloodstream circulate alongside red blood cells and are primarily taken up by the liver and spleen (*22*). Therefore, using exosomes as a delivery vehicle theoretically allows for the efficient targeting of liver tissues with effector molecules. In the current study, we prepared exosomes derived from mesenchymal stem cells loaded with miR-141-3p and applied them to human intrahepatic biliary epithelial cells (HIBECs) treated with LPS. We then assessed cell proliferation, migration capabilities, and the level of EGFR/ERK pathway expression to evaluate the effects of miR-141-3p-EVs and we further explored their potential mechanisms.

2. Materials and Methods

2.1. Cell culture

An HIBEC cell line was purchased from Otwo Biotech Company (Shen Zhen, China). HIBEC cells were cultured in RPMI-1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) and a 1% penicillin-streptomycin solution (Gibco, USA). All cells were maintained in a 5% CO2 atmosphere at 37 °C. The cells were subcultured when the confluence reached 80%. The current study used cells at passage 3. HIBEC cells were digested with 0.25% trypsin and resuspended in RPMI-1640 medium.

2.2. Isolation of EVs

Extracellular vesicles (dMSC-sEVs) were isolated and purified from the supernatant *via* ultracentrifugation. Initially, the MSC cell culture medium was centrifuged at 2,000 x g for 10 min to eliminate cell debris. The supernatant was then collected and centrifuged at 10,000 x g for 30 min. The new supernatant obtained underwent ultracentrifugation at 100,000 x g for 75 min. This supernatant was again collected and centrifuged at 10,000 x g for 30 min, followed by another ultracentrifugation of the new supernatant at 100,000 x g for 75 min. The resulting pellet was resuspended in 1 mL of PBS and filtered through a 0.22-μm filter. This suspension was then subjected to a final ultracentrifugation at 100,000 x g for 75 min. The dMSC-sEVs were resuspended in PBS and stored at -80 °C.

2.3. Preparation of miR-141-3p-loaded exosomes *via* electroporation

hsa-miR-141-3p (ID: MIMAT0000432) was purchased from GeneCopoeia. In a 60-mm dish containing 5 mL of exosome-free culture medium (with 50-70% cell confluence), 200 pmol of miR-141-3p or its inhibitor was mixed with 20 μg of exosomes in PBS, followed by the addition of CaCl2 to achieve a final concentration of 0.1 M. The final volume was adjusted to 300 μL using sterile PBS. The mixture was then incubated on ice for 30 min. After heat shocking at 42°C for 60 seconds, the mixture was placed back on ice for an additional 5 min. For RNase treatment of exosomes, the exosomes were incubated with RNase (5 μg/mL; EN0531, Thermo Fisher) at 37°C for 30 min. During electroporation, miR-141-3p was mixed with dMSC-sEVs at a 1:1 ratio (weight/weight) in electroporation buffer. The mixture was loaded into a Neon Tip and electroporated six times at 0.5 kV with 220-ms pulses, according to the manufacturer's instructions (Thermo Fisher Scientific).

2.4. Characteristics of EVs and MIR-141-3P-EVs

The size and concentration of EVs and miR-141-3p-EVs were measured using Nanoparticle Tracking Analysis (NTA) (Particle Metrix GmbH, Germany), and their morphology was observed using transmission electron microscopy (TEM).

2.5. Labeling of EVs

The membrane of HIBECs were labeled with PKH67 (green) and DAPI (cell nucleus, blue). EVs were counterstained with actin (cytoskeleton, red). Images were captured using a confocal microscope (Leica, Germany).

2.6. Cell proliferation

HIBEC cells (5×10^3 cells per well) were seeded into 96-well plates (Corning Inc, USA) and subjected to treatment with PBS, LPS (100 μg/mL), EVs (2×10^{10} particles/mL), and miR-141-3p-EVs (2×10^{10} particles/mL) for 24, 48, and 72 h. Optical density (OD) values were recorded at a wavelength of 450 nm using an automated plate reader. Cell proliferation was evaluated using the BeyoClick™ EdU Cell Proliferation Kit. After the cells were fixed with 4% paraformaldehyde and permeabilized with 0.3% Triton X-100 (Beyotime, China), they were sequentially incubated in the Click reaction mixture and Hoechst 33342 at room temperature, followed by imaging under a fluorescence microscope.

2.7. HIBEC cell migration assay

The migratory characteristics of HIBECs were evaluated using transwell and scratch assays. For the transwell assay, HIBECs were suspended in serum-free medium at a density of 5×10^3 cells/mL, and 100 μL of the cell suspension was seeded into the upper chamber of a 24-well plate with an 8.0-μm polycarbonate membrane (Corning Inc, USA). Different media were then added to the lower chamber. After the cells were cultured at 37°C with 5% CO2 for 24 h, migrating cells were stained with 0.1% crystal violet for 7 min. Finally, stained cells were observed under an optical microscope, and images were captured.

For the scratch assay, HIBECs were seeded into a 6-well plate. Once the cells reached 90% confluence, a pipette tip was used to scratch the cell layer. Images of the cells were captured using a microscope at 0 and 24 h post-scratch. The number of migrating cells and the distance between the two boundaries of the scratch were calculated using the software ImageJ. The migration rate was calculated as follows: Cell migration rate = (1 - width of the gap area (at 12 or 24 h) / width of the gap area (at 0 h)) × 100%.

2.8. Real-time reverse transcription polymerase chain reaction (RT-qPCR)

Total RNA was isolated from the miR-141-3p solution using the TRIzol® LS reagent (Invitrogen, Waltham, MA, USA). The quantity and quality of RNA samples were assessed using a Nanodrop 2000 (Thermo Fisher Scientific, San Jose, CA, USA) and RNA denaturing agarose gel electrophoresis. Complementary DNA (cDNA) was synthesized from RNA samples (OD260/280 ratio: 1.8-2.1; 28S/18S ratio: 2.0-2.5) using oligo (dT) and random primers (Thermo Fisher Scientific) and the Omniscript RT kit (Qiagen, Hilden, Germany). The RNA samples were further verified using RNA denaturing agarose gel electrophoresis. RNA expression was analyzed using the SYBR Premix Ex Taq™ II kit (Takara, Kusatsu, Japan), with cDNA prepared at a 1/4 dilution. The internal control primer HsnRNA U6 and the target primer hsa-miR-141-3p were purchased from GeneCopoeia. Standard curves were generated for each primer pair, and amplification efficiency was calculated using the formula E = 10^(-1/slope). Relative levels of RNA expression were calculated using the 2^-ΔCt method based on the threshold cycle (Ct) values.

2.9. Western blotting

Proteins extracted using RIPA buffer were separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then electrotransferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane was incubated with 5% non-fat milk for 1 h and then incubated overnight at 4°C with primary antibodies. These primary antibodies included anti-p-EGFR (1:1000, Abcam, USA), anti-GADPH (1:1000, Abcam, USA), anti-p-RAS (1:1000, Abcam, USA), anti-p38 (1:1000, Abcam, USA), anti-ERK-1 (1:1000, Abcam, USA), anti-MUC5AC (1:1000, Abcam, USA), and anti-p-ERK-1 (1:1000, Abcam, USA). After the membrane was washed, it was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:3000, ZSGB-BIO, China) at room temperature for 1 h. Antibody reactivity was detected using an ECL kit (Solarbio, China), and imaging was performed with the UVITEC Alliance MINI HD9 system (UVITEC, UK). The grayscale values representing levels of protein expression were quantified using the software ImageJ.

2.10. Creation of a mouse model of intrahepatic bile duct stone disease and treatment with miR-141-3p

All research plans and procedures were approved by the Institutional Animal Care and Use Committee. Male SD rats (BKS-Dock Leprem2Cd479, DB/db), aged 8 weeks, were purchased from SPF (Beijing, China) Biotechnology Co., Ltd. and were housed under standard laboratory animal conditions. Bile duct stone surgery was performed in the rat model as follows: a) The abdominal cavity was accessed by making a midline incision and gradually entering the peritoneal cavity layer by layer. b) The distal end of the common bile duct was isolated and silk suture was looped around it in preparation for ligation. c) The distal end of the common bile duct was ligated. d) Five 5 min were allowed to pass for the common bile duct above the ligation point to fill. e) A second ligature was placed above the first ligation point and a 24G cannula needle was used to puncture the common bile duct. After the rigid needle core was removed, the flexible needle was further advanced into the bile duct while ensuring that the second ligature remained within range of the needle body. f) The second ligature was tightened to secure the puncture needle. g) Point-two-five mg/mL of an LPS solution was injected at a dose of 0.5 mg/kg body weight. h) After the injection, the flexible needle was removed and the puncture site was ligated twice above the insertion point. i) The common bile duct was severed between the two ligatures placed in step (h) to induce bile duct obstruction. A total of 15 rats were randomly divided into three groups, with each group receiving different treatments *via* tail vein injection: a) PBS, b) dMSC-sEVs, and c) dMSC-sEVs-miR-141-3p. These treatments were administered to assess the therapeutic effects of dMSC-sEVs-miR-141-3p *in vivo*.

One week after induction of bile duct stones, bile samples were collected *via* common bile duct cannulation. The samples were centrifuged at 12,000 r/min for 10 min to remove the supernatant, and the

sediment was prepared for smearing. The experiment was conducted at a controlled room temperature of 25-30°C. The bile characteristics were observed using an Olympus microscope (polarized light). Additionally, the common bile duct, along with portions of the hepatic bile ducts and liver tissue, were excised for immunohistochemical analysis.

2.11. Histological analysis

Tissues from the bile duct were fixed in 4% paraformaldehyde, gradually dehydrated, and then embedded in paraffin. Five μm-thick sections of the embedded tissue were stained using the Hematoxylin and Eosin (H&E) Staining Kit (Solarbio, China) and Masson's Trichrome Staining Kit (Solarbio, China). For immunohistochemical staining, the sections, following deparaffinization, hydration, and antigen retrieval, were incubated overnight at 4 °C with primary antibodies anti-p-EGFR (1:1000, Abcam, USA), anti-GADPH (1:1000, Abcam, USA), anti-p-RAS (1:1000, Abcam, USA), anti-p38 (1:1000, Abcam, USA), anti-p-ERK-1 (1:1000, Abcam, USA), and anti-MUC5AC (1:1000, Abcam, USA). For immunohistochemistry, the sections were incubated with HRP-conjugated secondary antibody (1:200, Abcam, USA) at room temperature for 1 h. Immunocomplexes were visualized using the DAB Kit (ZSGBBIO, China). Images were recorded under a microscope and analyzed using the software Image-Pro-plus 6.0.

2.12. Statistical analysis

All data are expressed as the mean ± standard deviation. Comparisons between two groups were done using the unpaired Student's *t*-test, while comparisons among multiple groups were done using one-way ANOVA. A difference was considered statistically significant when $p < 0.05$.

3. Results

3.1 Preparation and characterization of EVs and miR-141-3p-EVs

EVs were isolated from the culture medium of mesenchymal stem cells. miR-141-3p was then incubated at 37°C and incorporated into the EVs using electroporation. Free miR-141-3p was removed by ultracentrifugation, and purified miR-141-3p-EVs were obtained by resuspending the pellet. TEM revealed that the EVs exhibited the characteristic cup-shaped and smooth bilayer structure, and they retained their original morphology after miR-141-3p incorporation (Figure 1A). Nanoparticle tracking analysis (NTA) indicated that the initial EVs had a diameter of 115.2 ± 1.7 nm, which increased to 136 ± 1.4 nm after miR-141-3p incorporation. In the saline control group, the median diameter of EVs was 141.6 ± 10 nm (Figure 1B). These changes in morphology and particle size are likely due to the encapsulation of miR-141-3p, as well as the incubation at 37°C and repeated ultracentrifugation steps. qPCR analysis of the miR-141-3p-EVs indicated a significantly higher level of miR-141-3p expression in the miR-141-3p-EVs compared to that in the NC-EVs group (Figure 1C). These findings confirm that the miR-141-3p-EVs meet the characterization standards for extracellular vesicles, consistent with their potential as a next-generation drug delivery platform. Uptake experiments demonstrated that, after 12 h, EVs from both

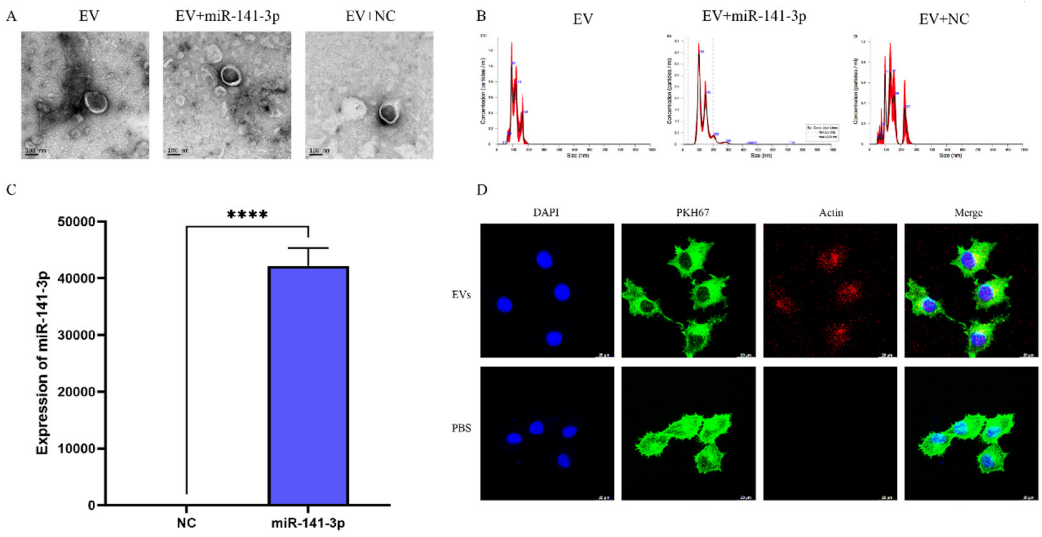

Figure 1. Preparation and characterization of EVs and miR-141-3p-EVs. (A) TEM images showing the morphology of EVs, EV-miR-141-3p, and EV-NC. **(B)** NTA results indicating the particle size distribution of exosomes in different groups. **(C)** Levels of miR-141-3p expression in miR-141-3p-EVs. **(D)** Exosome uptake assay: EV membranes were labeled with actin (red), HIBEC cell membranes were labeled with PKH67 (green), and nuclei were labeled with DAPI (blue).

groups (PKH26, red fluorescence) were successfully transferred into HIBECs (HIBEC cell membranes labeled with PKH67, green fluorescence; nuclei labeled with DAPI). As shown in Figure 1D, there was no difference in the uptake rates of the two groups, suggesting that the observed differences in biological function are due to the different compounds encapsulated within the vesicles.

3.2. miR-141-3p-EVs downregulate MUC5AC expression and inhibit intrahepatic bile duct stone formation

The proliferative capacity of HIBEC cells under different treatment conditions was assessed using the EdU assay (Figure 2). Green fluorescence indicates proliferating HIBEC cells. LPS was the most potent promoter of cell proliferation, while HIBEC cells treated with miR-141-3p-EVs displayed a significant reduction in proliferation compared to the group treated with exosome analogs (Figure 2, A and B). The migratory ability of HIBEC cells under different culture conditions was evaluated using a scratch assay. Scratch assays were performed 24 h after treatment with PBS, LPS, EVs, or miR-141-3p-EVs. HIBEC cells treated with miR-141-3p-EVs exhibited a significantly lower scratch closure rate after 24 h compared to other groups (Figure 2, C and D). A transwell assay further indicated that the LPS group had the largest migration area of HIBEC cells while those treated with miR-141-3p-EVs had the smallest migration area, and it differed significantly compared to that in the LPS group (Figure 2, E and F).

3.3. miR-141-3p-EVs regulate MUC5AC expression *via* the MAPK pathway

Western blotting indicted that the expression of MAPK pathway proteins was consistently downregulated in HIBEC cells treated with AG1478, and miR-141-3p-EVs demonstrated more potent ability to downregulate MUC5AC gene expression in HIBEC cells (Figure 3A). In contrast, the levels of MAPK and MUC5AC protein expression in HIBEC cells treated with EVs or miR-141-3p-EV inhibitors did not decrease significantly. This experiment confirmed that miR-141-3p-EVs inhibit the formation of intrahepatic bile duct stones by downregulating the MAPK pathway and reducing levels

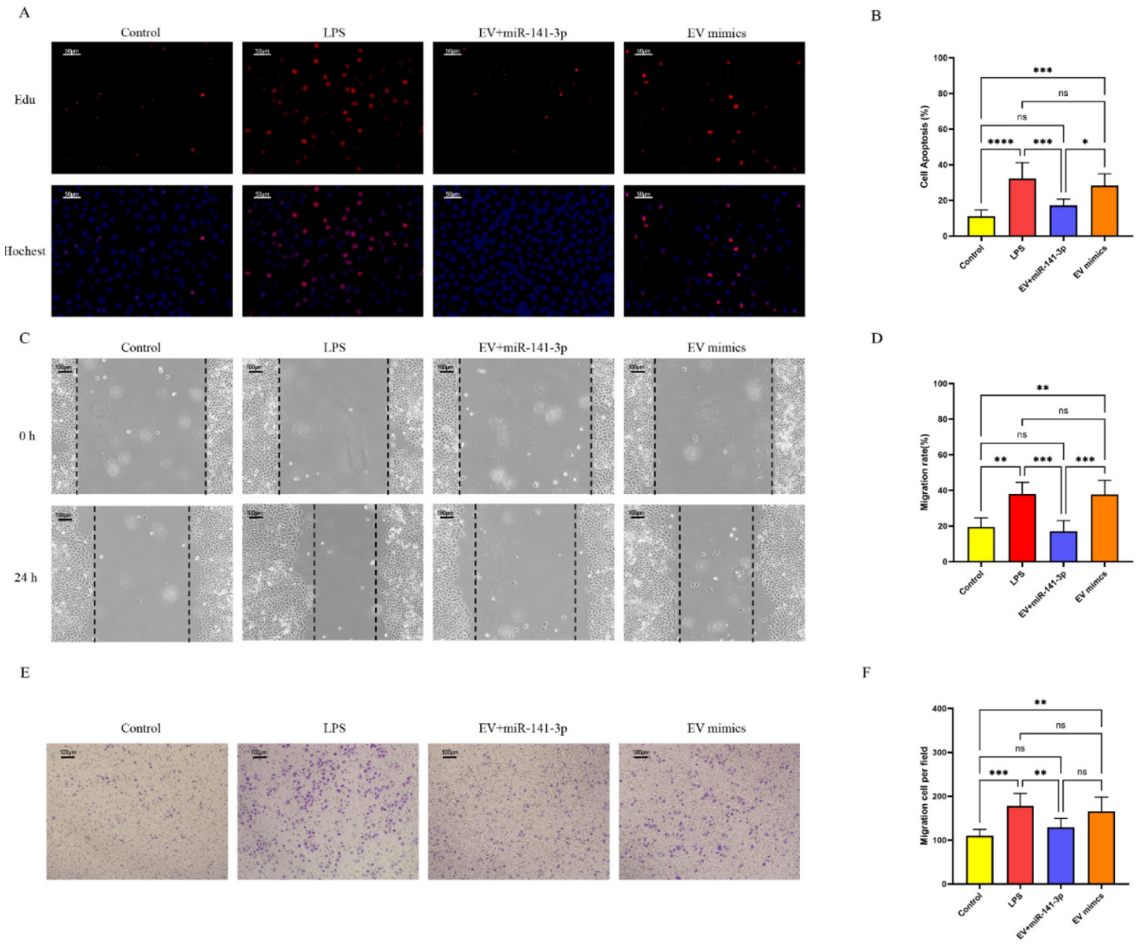

Figure 2. miR-141-3p-EVs inhibit an LPS-induced inflammatory response and proliferation in HIBECs. (A) EdU assay (EdU-positive cells are labeled green, and nuclei in each group are labeled in red). **(B)** Quantification of the rate of EdU-positive cell proliferation (n = 7). **(C)** Scratch assay (images taken at 0h and 24h post-scratch). **(D)** Statistical analysis of the scratch closure area (n = 7). **(E)** Representative images of the transwell assay of HIBECs in different groups. **(F)** Quantitative analysis of HIBEC migration in the transwell assay.

of MUC5AC expression.

3.4. Effect of miR-141-3p-EVs on intrahepatic bile duct stone formation in rats

In animal experiments (Figure 4), dMSC-sEVs-miR-141-3p had a positive therapeutic effect on rats with induced intrahepatic bile duct stones. Observations of rat bile under polarized light microscopy revealed that the bile in the dMSC-sEVs-miR-141-3p group was noticeably thinner, with significantly fewer bile stones formed compared to those in the control group.

In the immunohistochemical experiment (Figure 5), the bile duct tissue of rats in the dMSC-sEVs-miR-141-3p group displayed significantly lower levels of EGFR, p-ERK, RAS, and MUC5AC expression compared to those in the LPS group and the LPS+EV+EV inhibitor group. These decreases were significant.

4. Discussion

This study successfully prepared exosomes loaded with miR-141-3p and validated their stable and reliable drug delivery properties. Findings indicated that miR-141-3p-EVs can reduce LPS-induced inflammatory responses in intrahepatic biliary epithelial cells and, to some extent, inhibit the formation of intrahepatic bile duct stones. *In vitro* studies demonstrated that LPS promotes inflammatory responses in HIBECs while miR-141-3p-EVs alleviate these LPS-induced inflammatory responses by downregulating the MAPK pathway. *In vivo* experiments confirmed that intravenous administration of miR-141-3p-EVs effectively reduces bile duct stone formation and decreases the level of MAPK pathway expression in biliary epithelial cells.

Previous studies have confirmed that LPS induces the overexpression and secretion of MUC5AC *via* the EGFR/ERK pathway. The polymerized form of MUC5AC, along with its abundant O-linked oligosaccharides, increases bile viscosity, thereby accelerating stone formation. Additionally, MUC5AC can constitute a major component of bile duct stones, aggregating bile constituents such as bilirubin crystals and desquamated epithelial cells to form the core and scaffold of calcium bilirubinate stones (*23,24*). Therefore, targeting the EGFR/ERK pathway-mediated overexpression of MUC5AC may inhibit this pathway, preventing stone formation and recurrence, and it could effectively reduce the incidence of intrahepatic bile duct stones. This study developed mesenchymal stem cell-derived exosomes loaded with miR-141-3p. Experiments demonstrated that miR-141-3p effectively downregulates the expression of the EGFR/ERK pathway and MUC5AC, leading to a reduction in the formation of intrahepatic bile duct stones.

MicroRNAs (miRNAs) are a class of endogenous small RNAs, approximately 20-24 nucleotides in length, which play critical regulatory roles in disease processes. Each miRNA can target multiple genes, and several miRNAs can regulate the same gene. Previous studies have shown that miR-141-3p can negatively regulate the EGFR/ERK pathway, thereby inhibiting the progression of inflammation or tumors. For instance, Xing *et al.* found that miR-141-3p suppresses the proliferation, migration, and invasion of colorectal cancer cells by downregulating the EGFR/ERK pathway

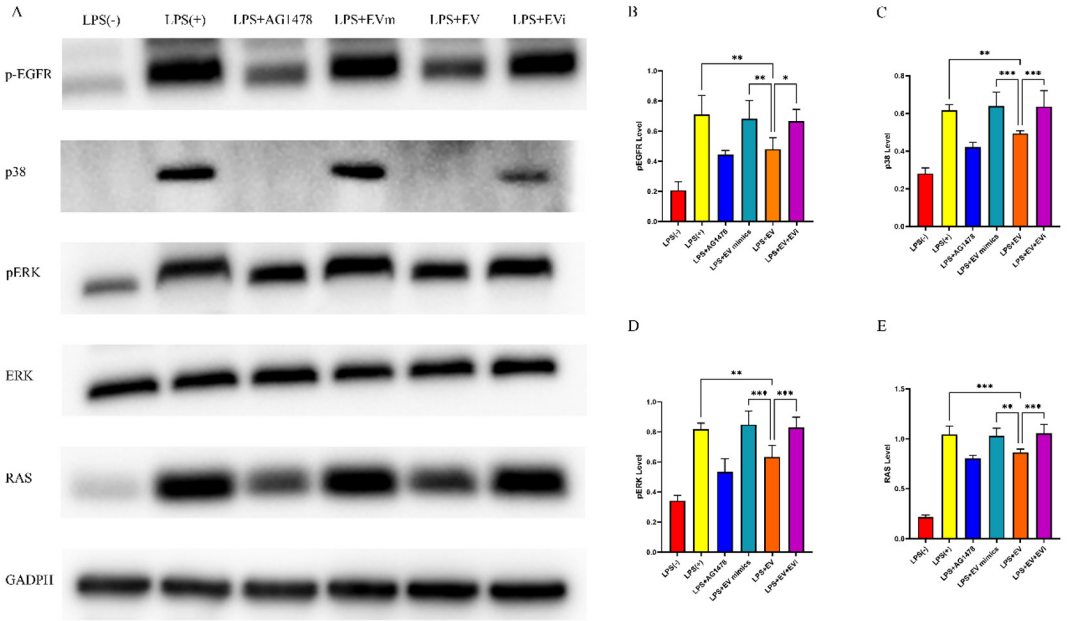

Figure 3. (A) Representative Western blotting images showing the expression of EGFR, p38, p-ERK1, RAS, and GAPDH in HIBECs under different treatment conditions: PBS, LPS, LPS + AG1478, LPS + EV mimics, LPS + EV, and LPS + EV + EVi. **(B-E)** Relative protein levels in each group, with p-EGFR, p38, and RAS normalized to GAPDH and p-ERK normalized to ERK.

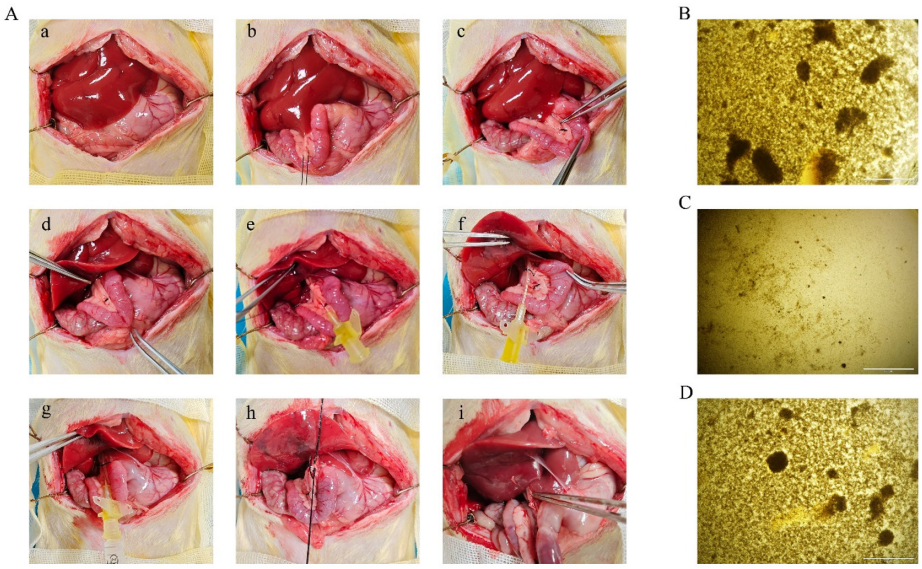

Figure 4. Bile duct stone surgery in a rat model and bile microscopy from different groups. (A) Images of bile duct stone surgery in a rat model: **(a)**: Midline abdominal incision to access the abdominal cavity. **(b)**: Isolation of the common bile duct with silk suture looped around it for marking. **(c)**: Ligation of the distal end of the common bile duct. **(d)**: Visible bile duct obstruction and dilation above the ligation point. **(e)**: Placement of a pre-ligation suture and insertion of a 24G cannula needle above the ligation point. **(f)**: Tightening of the pre-ligation suture to secure the cannula needle, with bile leakage indicating successful puncture. **(g)**: Connection of a syringe and injection of 0.25 mg/mL LPS solution. **(h)**: Removal of the cannula needle and ligation of the common bile duct above the puncture site. **(i)**: Severing of the ligated common bile duct, resulting in obstruction and dilation above the ligation site. **(B)** Bile characteristics in rats injected with saline *via* the tail vein. **(C)** Bile characteristics in rats injected with dMSC-sEVs-miR-141-3p *via* the tail vein. **(D)** Bile characteristics in rats injected with dMSC-sEVs-miR *via* the tail vein.

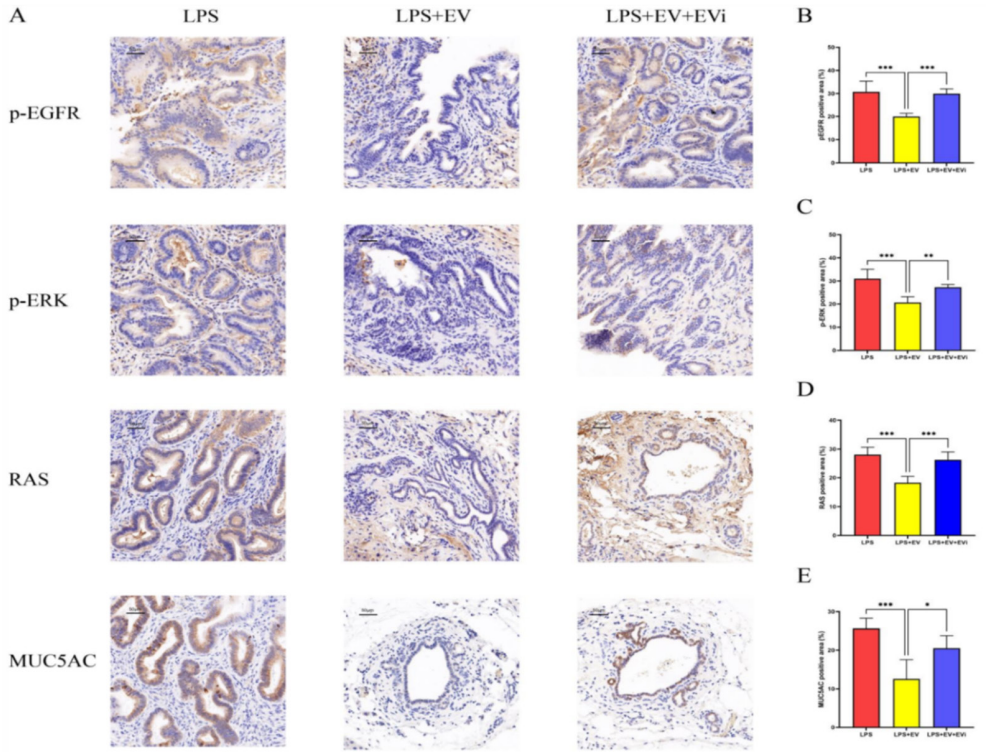

Figure 5. Immunohistochemical results in rat bile duct tissue. (A) Immunohistochemical staining of rat bile duct tissue. **(B)** Percentage of p-EGFR-positive areas. **(C)** Percentage of p-ERK-positive areas. **(D)** Percentage of RAS-positive areas. **(E)** Percentage of MUC5AC-positive areas.

(*13*). Similarly, Xue *et al.* reported that the bioceramic sealer iRoot SP promotes osteogenic differentiation of hSCAPs by inhibiting miR-141-3p, downregulating SPAG9 expression, and activating the MAPK pathway (*25*). Additionally, the antagonistic effects of miR-141-3p against LPS have also been increasingly explored. Xia *et al.* reported that miR-141-3p can counteract LPS-induced apoptosis and inflammatory responses in human lung fibroblasts (*14*). Similarly, Zhu *et al.* found that miR-141-3p can inhibit apoptosis and the expression of MUC5AC in LPS-pretreated nasal mucosal cells (*15*) . As previously discussed, LPS-induced overexpression of MUC5AC plays a critical role in the formation of intrahepatic bile duct stones. Therefore, we hypothesize that miR-141-3p could be a novel target for the prevention and treatment of intrahepatic bile duct stones by downregulating MUC5AC expression *via* the EGFR/ERK pathway. Western blotting revealed that the expression of the EGFR/ERK pathway and MUC5AC in HIBEC cells stimulated with LPS increased significantly compared to that in the PBS group, and this is consistent with the mechanism by which LPS induces intrahepatic bile duct stone formation. However, exosomes loaded with miR-141-3p effectively reduced the expression of EGFR, ERK, RAS, and MUC5AC. In contrast, no negative regulation of the EGFR/ERK pathway was observed in groups treated with either exosomes alone or exosome inhibitors, suggesting that the downregulation of MUC5AC expression was mediated by miR-141-3p. A transwell assay indicated that miR-141-3p-EVs significantly reduced the migratory ability of LPS-treated HIBEC cells, further indicating that miR-141-3p lowers the inflammatory response in HIBEC cells. Similar results were observed in the scratch assay, where miR-141-3p-EVs reduced cell migration. Additionally, an EdU assay showed that HIBEC cells treated with miR-141-3p-EVs exhibited decreased proliferation compared to LPS-treated HIBEC cells. Since the EGFR/ERK pathway is closely associated with cell proliferation, the negative regulation of EGFR expression by miR-141-3p likely led to the observed suppression of HIBEC cell proliferation. These findings further confirm the effectiveness of miR-141-3p-EVs in negatively regulating EGFR expression.

In this study, Sprague-Dawley (SD) rats were used as experimental animals to establish an intrahepatic bile duct stone model. Based on research by Chang *et al.,* LPS was injected into the bile ducts to induce the formation of intrahepatic bile duct stones in the rabbits *(26)*. The model was successfully created based on the characteristics of bile and stone morphology observed under a microscope. In the animal experiments, the bile duct tissues of rats injected with miR-141-3p-EVs exhibited significantly lower expression of EGFR, ERK, and MUC5AC proteins in immunohistochemical analyses, whereas rats injected with EV mimics showed minimal downregulation of EGFR, ERK, and MUC5AC expression. This result was further validated by immunohistochemical findings in rat bile duct tissues, where intravenous injection of miR-141-3p-EVs notably reduced the expression of the MAPK pathway in the intrahepatic bile ducts and effectively decreased MUC5AC secretion, thereby inhibiting the formation of intrahepatic bile duct stones. Based on both *in vivo* and *in vitro* experiments, we conclude that EVs loaded with miR-141-3p can effectively reduce the expression of the EGFR/ERK pathway and further decrease MUC5AC secretion, achieving a therapeutic effect against intrahepatic bile duct stones.

We developed miR-141-3p-loaded exosomes as a novel therapeutic approach for treating intrahepatic bile duct stone disease. Exosomes are naturally produced by cells and are small vesicles that are highly biocompatible with the human body and have a low level of immunogenicity, making them less likely to trigger a strong immune response. MicroRNAs are prone to degradation in natural environments, but exosomes can stably carry RNA and other macromolecular drugs, protecting them from degradation by enzymes both outside and inside the body. Due to their targeting ability and low level of immunogenicity, RNA drugs delivered *via* exosomes may cause fewer adverse reactions than traditional delivery methods. In this study, use of exosomes as a delivery vehicle for miR-141-3p provided a safer, more effective, and targeted method of treatment, offering new insights into the development of novel drugs for the treatment of intrahepatic bile duct stone disease.

However, this study did had a limitation. The reduction of MAPK pathway activity in human tissues by miR-141-3p was not observed or investigated. Further studies are needed to explore the efficacy and safety of miR-141-3p-loaded exosomes in the treatment of intrahepatic bile duct stone disease.

In conclusion, we developed an exosomal drug harboring miR-141-3p, which alleviates intrahepatic bile duct stone disease by inhibiting the expression of the EGFR pathway in intrahepatic bile duct epithelial cells. It also demonstrated preliminary therapeutic effects for intrahepatic bile duct stone disease and has the potential to become a clinical therapeutic drug.

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Brief Report

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A novel ETFDH mutation identified in a patient with riboflavin-responsive multiple acyl-CoA dehydrogenase deficiency

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SUMMARY: Lipid storage myopathies (LSM) are a group of inherited metabolic muscle disorders characterized by abnormal lipid metabolism and the deposition of lipids within muscle fibers. Multiple acyl-coenzyme A dehydrogenase deficiency (MADD) is the most common type of LSM in China, caused by mutations in the gene expressing electron transfer flavoprotein dehydrogenase (*ETFDH*). Here, we report a 14-year-old girl presenting with exercise intolerance, followed by muscle weakness and pain. Initially, the patient showed rhabdomyolysis (RML) and was misdiagnosed with polymyositis (PM). However, muscle biopsy and genetic analysis led to a diagnosis of MADD. After the initiation of vitamin B2 administration, her symptoms were rapidly ameliorated. Genetic testing revealed compound heterozygous mutations in the *ETFDH* gene, specifically c.250G>A and c.929A>G, the second of which has not previously been reported. In conclusion, we report a novel mutation of *ETFDH* in a patient with riboflavin-responsive MADD, which expands our knowledge of MADD-related gene variants in the Chinese population.

Keywords: MADD, *ETFDH* gene, magnetic resonance imaging, RML, PM

1. Introduction

Multiple acyl-coenzyme A dehydrogenase deficiency (MADD) is an autosomal recessive genetic disorder caused by mutations in the *EFTA*, *EFTB*, or *ETFDH* (expressing electron transfer flavoprotein dehydrogenase) genes. The most common symptoms of MADD are muscle weakness, exercise intolerance, and muscle pain (*1*), and the signs of MADD include poor muscle strength and weak head lifting (*2*). Patients with late-onset MADD typically exhibit imaging findings of fatty infiltration and atrophy in the anterior, posterior, and medial muscle groups of the thigh on muscle magnetic resonance imaging (MRI) (*3*). Blood acylcarnitine analysis typically reveals high concentrations of medium- and long-chain acylcarnitines (*4*), and serum creatine kinase (CK) activity is often high, although this is not specific to MADD (*5*). The key pathologic feature of MADD is the deposition of fat within muscle fibers (*6*). MADD can easily be misdiagnosed as polymyositis (PM) (*7*), and patients with MADD rarely present with Rhabdomyolysis (RML) (*8*). Some patients show a significant amelioration of their symptoms after treatment with riboflavin, and these are referred to as having riboflavin-responsive (RR)-MADD (*9*).

This study retrospectively analyzed a case of a patient with MADD, attempting to elucidate the differentiation of MADD from PM and RML based on early clinical manifestations and serum CK activity. We followed up with the patient's muscle MRI and observed the resolution process of muscle edema-signals in this MADD patient. Meanwhile, this study analyzed the *ETFDH* gene in a MADD patient using Sanger sequencing, and the results revealed a novel *ETFDH* mutation, further expanding the genetic data associated with this disease.

2. Patient and Methods

2.1. Patient

This was an opportunistic study, but the patient and her parents were informed about the possible use of the data for research and the purpose of the study, the methods used, and gave their consent. The patient was able to understand the study, regardless of her age, was informed and gave her consent. The study conformed to the provisions of the latest version of the Declaration of Helsinki.

2.2. Clinical data collation and analysis

We collected information from the patient and her parents about the symptoms of the disease, and the history of diagnosis and treatment, past medical history, genetic history, *etc*. After sorting out the clinical data and analysis, the next step of the diagnosis and treatment plan was launched.

2.3. Muscle biopsy and pathology

We first took a total of about 0.3 × 0.3 × 0.6 cm of skeletal muscle tissue, performed frozen sections, and completed histochemical and immunohistochemical staining. In addition, two pieces of glutaraldehyde fixed gray and yellow tissue, about 0.2 × 0.3cm long, were taken and a box was used for electron microscopy.

2.4. Sanger sequencing

Sanger sequencing was applied to validate the mutation of the *ETFDH* gene for the patient and her parents. Gene mutation analysis was performed on the DNA sequences of exons 3 and 8 of *ETFDH* gene of the patient.

3. Results and Discussion

3.1. Review and analysis of medical history

A 14-year-old girl presented with generalized muscle weakness and pain, along with a 3-year history of exercise intolerance. Her condition gradually worsened, weakness in lifting her head and chewing developed, and this was accompanied by respiratory distress, necessitating non-invasive ventilation. Her CK activity was as high as 88,703 U/L (reference range: 40-200 U/L). She was initially diagnosed with PM and RML at another hospital, underwent treatments including blood dialysis and immunoglobulin and methylprednisolone administration. However, her symptoms were not significantly ameliorated.

After the patient was admitted to our hospital, we completed muscle biopsy and genetic screening. Soon, the patient was definitively diagnosed with MADD. Then she was prescribed oral vitamin B2. Two months later, she was able to walk normally and perform simple physical activities, such as squatting and standing up repeatedly.

When we retrospectively analyzed this case, we considered whether it was possible to differentiate MADD from PM and RML through early clinical manifestations. The early clinical manifestations of this patient were similar to those of MADD and PM, along with extremely high CK activity. We could not rule out whether she had RML at the onset of the disease. Given the limitations of our case, we reviewed the literature. Torres *et al*. stated that a combination of MADD and RML is extremely rare, and RML is more commonly associated with viral myositis, polymyositis, and other muscle diseases (*8*). In addition, there was a scholar who attempted to distinguish between MADD and PM based on clinical manifestations. Wang *et al*. showed that the symptoms of this disease have more variability in muscle weakness, significant involvement of the muscles of mastication, fewer extra-muscular manifestations, and lower CK activity, in the absence of RML. Under these circumstances, MADD should be considered the primary diagnosis, rather than PM (*10*).

However, due to the extremely high CK level in the early stage of this patient, we were unable to determine whether the abnormal increase in CK was caused by the patient's own condition or was due to concurrent RML. Moreover, during the diagnostic process of a single patient, it is difficult to distinguish between PM and MADD solely based on clinical manifestations. Therefore, we believe that in the early stage, it is challenging to differentiate muscle-related diseases with similar symptoms through clinical manifestations and test results alone. Muscle biopsy and genetic screening should be completed as early as possible.

3.2. Analysis of muscle MRI

Our patient's muscle MRI of both lower extremities showed edema - like changes in multiple muscle groups of the lower limbs before treatment (Figure 1, A and B), but did not show typical fatty infiltration imaging findings of MADD (*3*). 1 month later, the symptoms of this patient were significantly improved, but the MRI did not change significantly (Figure 1, C and D). One year later, we re-examined the MRI of both ankles and found that the edema of the bilateral soleus muscles, the right tibialis anterior muscle, and the right posterior tibialis muscle was less than before (Figure 1, E and F).

By contrast, Hong *et al.* found that some patients showed edematous changes and fatty infiltration in the soleus and biceps femoris muscles on muscle MRI before treatment. After 1 month of treatment, these edematous changes rapidly resolved and the symptoms were alleviated (*11*). Therefore, based on the results of our patient, not all MADD patients will show fatty infiltration on muscle MRI. Moreover, the resolution of abnormal signals on muscle MRI may not be consistent with the improvement of clinical manifestations. In the future, whether the abnormal muscle manifestations can actually serve as an indicator for the prognosis of the disease requires reference and research from more clinical cases.

3.3. Muscle pathological results

Muscle biopsy revealed the presence of vacuoles and lipid droplets in muscle fibers, predominantly in type I muscle fibers, which is consistent with the pathologic characteristics of MADD (Figure 2).

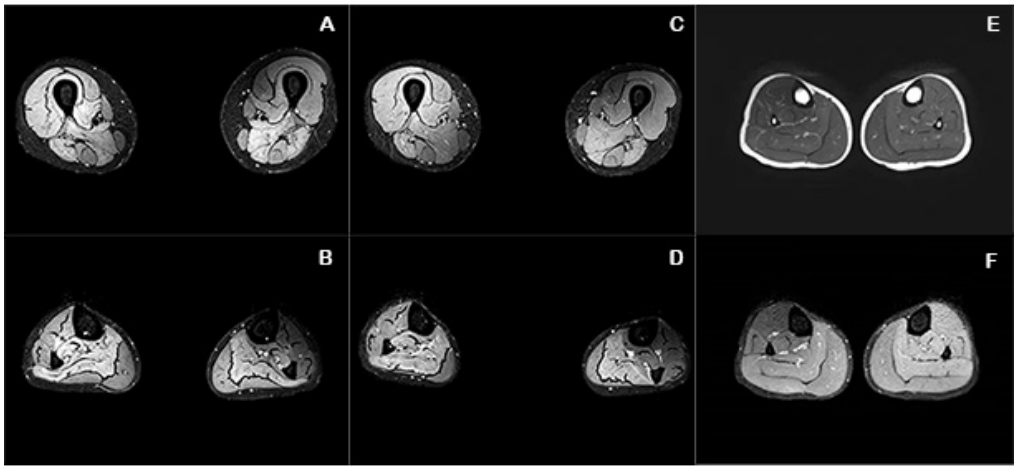

Figure 1. Muscle magnetic resonance imaging findings before and after riboflavin treatment. (A, B) Before treatment, the patient exhibited abnormal edema - like signals in both lower limbs; **(C, D)** After one - month treatment, these signals had not significantly changed; **(E, F)** A year later, the bilateral calf muscle edema became less obvious.

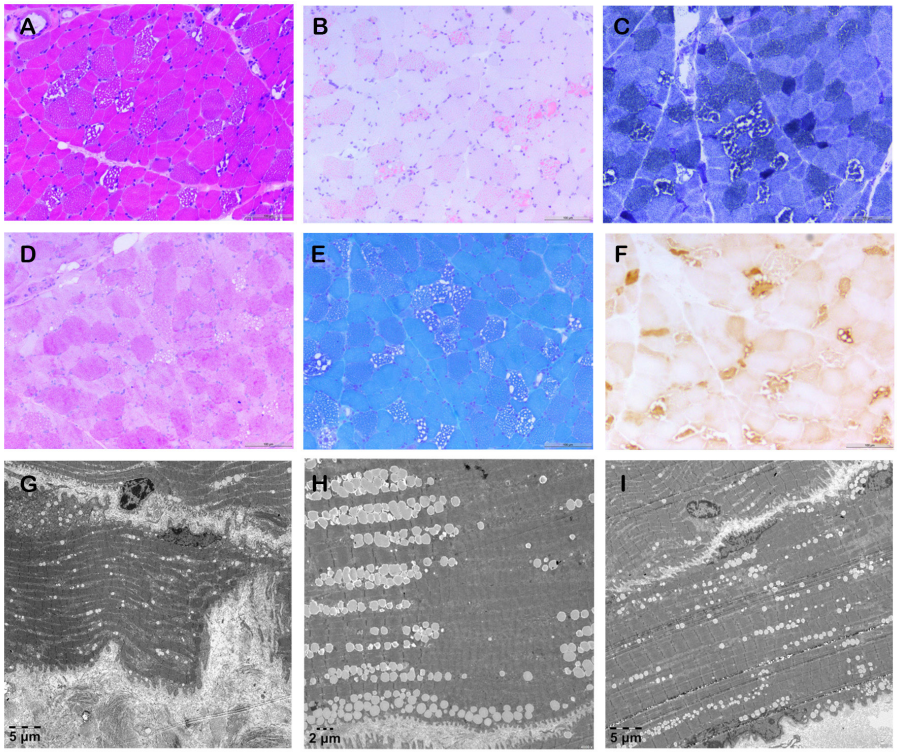

Figure 2. Muscle histopathologic findings. (A) Hematoxylin and eosin staining revealed abundant small vacuoles scattered within muscle fibers, with some showing fusion to form larger vacuoles; **(B)** Oil red O staining revealed diffuse lipid deposition within muscle fibers; **(C)** NADH staining demonstrated a mixed fiber-type distribution, with scattered atrophic fibers appearing darker against a lightly stained background; **(D)** PAS staining did not reveal significant glycogen accumulation within muscle fibers; **(E)** MGT staining did not show fragmentation of red fibers or rimmed vacuoles; **(F)** Deep COX staining was present in scattered atrophic muscle fibers against a lightly stained background, with no COX-negative fibers; **(G, I)** Electron microscopy revealed numerous lipid droplets arranged in a "beads-on-a-string" pattern within muscle fibers.

3.4. Analysis of gene screening results

Genetic sequencing identified compound heterozygous mutations in the *ETFDH* gene of the patient, with c.250G>A having been inherited from her father and c.929A>G from her mother (Figure 3).

In China, most patients with LSM have late - onset MADD caused by mutations in the *ETFDH* gene (*12*). The most frequent site of mutation in Southern China is c.250G>A (*13*). Our patient had both c.250G>A (p.A84T) and c.929A>G (p.Y310C) compound heterozygous mutations, and c.250G>A (p.A84T) has previously been reported to be a pathogenic mutation (*14*). In addition, we entered the following search terms

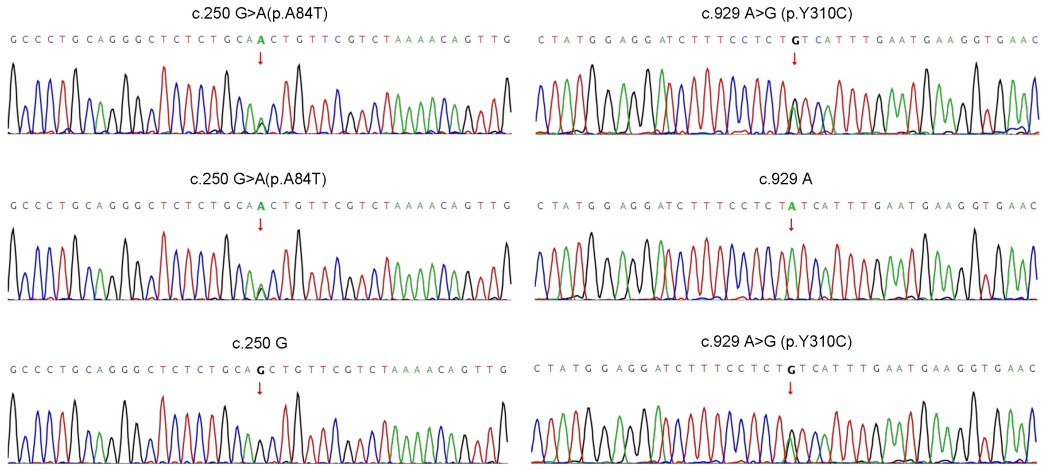

Figure 3. Genetic analysis of the *ETFDH* gene. (FIRST LINE) The genetic analysis suggested the presence of compound heterozygous mutations (c.250G>A and c.929A>G) in the *ETFDH* gene of the patient. **(SECOND LINE)** The c.250G>A mutation had been inherited from her father; **(THIRD LINE)** the c.929A>G mutation had been inherited from her mother.

in the Advanced function of PubMed: (c.929A>G) AND (Multiple acyl - coenzyme A dehydrogenase deficiency); (c.929A>G) AND (MADD). There are no relevant reports in the literature indicating that it is a pathogenic mutation. To our knowledge, nor has it been reported as a benign polymorphism.

We attempted to interpret the newly discovered mutation and identified the following consequence: The amino acid change is semi-conservative as both Tyrosine and Cysteine are uncharged, polar amino acids, but the introduction of a Cysteine could affect the disulfide bonds of the *ETFDH* protein. This change is at a highly conserved position in the ETFDH protein, and multiple *in-silico* analysis programs predicted that Y310C would be damaging to the ETFDH protein. Therefore, Y310C can be interpreted as a pathogenic mutation.

4. Conclusion

We have discussed the rarity of MADD in clinical practice and the complexity of making a clinical diagnosis. Clinical diagnosis should involve early muscle biopsy and genetic sequencing. We reported a novel *ETFDH* mutation in a patient with RR - MADD, expanding knowledge of the genetic spectrum of MADD in the Chinese population.

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Herpes zoster central nervous system complication: An increasing trend of acute limbic encephalitis

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SUMMARY: Varicella zoster virus (VZV) causes chickenpox as the primary infection and then becomes latent in the cranial and spinal ganglia. VZV can reactivate with aging, immunosuppression, stress, and other factors. In our series of 15 patients with herpes zoster (HZ) central nervous system complications (8 males and 7 females, ages 41-86 years), we identified several types of complications: acute encephalitis, vasculitis, meningitis, and cranial nerve palsies, with acute limbic encephalitis (ALE) (n = 5) being particularly noteworthy. The elderly patient treated initially showed skin rash around the eye, altered consciousness, and medial temporal lesions on MRI; four similar patients were then observed. Aside from a few case reports, there are no comprehensive reports of HZ ALE. The HZ rashes of our five HZ ALE patients were mostly in the trigeminal nerve area, with two cases of disseminated rashes. Five patients had positive cerebrospinal fluid VZV polymerase chain reaction results, and MRI revealed medial temporal lobe lesions. Compared to HZ peripheral nerve complications, more variable invasive routes were presumed, *via* the brain-stem, vasculopathy root, meningeal spread, and viremia. The incidence of HZ is increasing worldwide, and clinicians should be aware of HZ ALE that shows fever, HZ skin rash, and altered consciousness.

Keywords: herpes zoster, varicella-zoster virus, cranial nerve ganglia, acute limbic encephalitis, limbic system

1. Introduction

Varicella zoster virus (VZV) causes varicella as the primary infection and then becomes latent in trigeminal and spinal ganglia (*1,2*). Herpes zoster (HZ) central nervous system (CNS) complications caused by VZV reactivation include acute encephalitis, cerebral infarction due to vasculitis, meningitis, and cranial nerve palsies, and cases of patients with HZ CNS complications have been accumulating from medical departments such as internal medicine, dermatology, ophthalmology, and otolaryngology (*3-5*). In an aging society, HZ is more prevalent in adults and elderly persons with immunosuppression (*6*), and these individuals' complications can severely impair one's quality of life (QOL) and make long-term hospitalization necessary.

In our present retrospective analyses of the cases of 15 patients with HZ CNS complications who were treated at our hospital in 2012-2023, it was particularly noteworthy that five of the patients had acute limbic encephalitis (ALE). We first observed an 86-year-old woman with Alzheimer's disease who presented with an HZ skin rash on the trigeminal nerve area, impaired consciousness, and seizures. Her cerebrospinal fluid (CSF) was positive in a VZV polymerase chain reaction (PCR), and magnetic resonance imaging (MRI) revealed medial temporal lobe lesions (*7*). Four similar patients with ALE were encountered after this patient. The invasive routes of CNS complications from VZV reactivation in the trigeminal ganglia are presumed to be *via* the brainstem or carotid artery or meningeal spread.

Our search of the relevant literature identified no comprehensive reports of HZ ALE, and the pathophysiology is not clear. We report results of our retrospective analysis of mainly five patients with ALE among 15 patients with HZ/VZV, focusing on the patients' underlying diseases, vaccination history, skin rash distribution, virological test results such as CSF VZV PCR, MRI findings, treatments, and related disorders.

2. Research design and data collection

From April 2012 to March 2023, 15 patients with

HZ CNS complications such as acute encephalitis, meningitis, vasculitis, and others were treated at the departments of cerebrovascular medicine and neurology at our hospital in Fukuoka, Japan. We retrospectively analyzed the patients' cases based on their hospitalization histories concerning underlying disease, HZ skin rash, neurological form, length of hospitalization, CSF test results, CSF VZV PCR results, VZV antibody findings, MRI results including contrast-enhanced fluid attenuated inversion recovery (FLAIR) images, and the time course of these findings, as well as treatment(s) and outcomes.

The diagnostic criteria for HZ ALE are the presence of: fever, rash, impaired consciousness, abnormal behavior, seizures, CSF VZV PCR-positivity, and MRI results exhibiting medial temporal lobe lesions. Cases in which ALE was clinically suspected but MRI findings were unclear were classified as possible ALE. In several cases, electroencephalography (EEG) findings were examined. We considered patient cases lacking localized brain symptoms and showing fever, headache, meningeal irritation symptoms, and an increased CSF cell count as having meningitis. This study was conducted in accord with the Declaration of Helsinki and was submitted to and approved by our Hospital's Ethics Committee (Research 24-0505).

3. Key research findings

Our hospital is located in southwestern Japan and provides emergency medical care for ~300,000 local residents. Table 1 summarizes the patients' clinical characteristics. The mean age of the eight males and seven females was 74.1±10.3 years (range 41-86 years). The neurologic forms were classified according to the above diagnostic criteria: ALE (n = 5, including one patient with possible ALE), acute encephalitis (n = 3), cerebellitis (n = 1), meningitis (n = 5), and multiple cranial nerve palsies (n = 1). The patients' underlying diseases were hypertension (n = 9), dyslipidemia (n = 3), Alzheimer disease (n = 2), lumbar deformity (n = 2), colon cancer (n = 1), prostatic hypertrophy (n = 1), and others. Regarding the HZ dermatome, we identified the trigeminal region (first branch n = 6, second n = 2), VIII region (n = 1), VIII + generalized (n = 2), cervical region + generalized (n = 1), varicella rash (n = 1), thoracic region (n = 1), and sacral region (n = 1).

The hospitalization period was 32.5±10.3 days, and ten patients required a 1-month hospitalization at the acute stage for antiviral therapy. Average length of hospital stay for the five patients with ALE was 36.0±3.7 days, which is longer compared to the other types. An increase in the CSF cell number > 5 cells was observed in all 15 patients (mean 73.5 cells, range 7-381/μL). The VZV PCR result for the CSF was positive in 13 patients. All patients were intravenously administered acyclovir 500 mg 3×/day for 2-3 weeks, and 3 days of prednisolone pulse was added for three patients.

Table 1. Clinical characteristics of the 15 patients with herpes zoster central nervous system complications treated in 2012-2023

Clinical characteristics	Patients, *n* (%).
Age, years, mean ± SD, gender	74.1 ± 10.3 years 8 males, 7 females
Underlying disease	
Hypertension	9 (60%)
Alzheimer type dementia	2 (13%)
Malignancy	2 (13%)
Diabetes	2 (13%)
Other	5 (33%)
Herpes zoster	
V-1, V-2	6, 2 (40, 13%)
VIII, VIII + generalized	1, 2 (7, 13%)
C + generalized	1 (7%)
Varicella rash	1 (7%)
Th, S	1, 1 (7, 7%)
Neurologic form	
Acute limbic encephalitis	5 (33%)
Acute encephalitis	3 (20%)
Cerebellitis	1 (7%)
Meningitis	5 (33%)
Cranial nerve palsies	1 (7%)
Cerebrospinal fluid	
Cell count, /μL mean (range)	73.5 ± 79.5 (7-381)
VZV PCR positive	13/15 cases positive
Blood VZV PCR positive	1/15
Treatment:	
ACV IV	2-3 weeks
VCV IO	
PLS pulse, 3 days	3 (20%)
Hospitalization, mean (range)	32.5 ± 10.3 days (16-52)
Acute limbic encephalitis, mean (range)	36.0 ± 3.7 days (31-45)
Sequelae	
Post-herpetic neuralgia	5 (33%)
Cognitive disability	4 (27%)
Bed-patient with full assistance	2 (13%)
Non	4 (27%)

ACV IV: acyclovir intravenous, C: cervical, PLS pulse: prednisolone pulse, PCR: polymerase chain reaction, S:sacral, Th: thoracic, VCV: valacyclovir intraoral, V: trigeminal, VII/VIII: facial/acoustic.

It is noteworthy that five patients with ALE were identified among 15 patients with VZV reactivation and CNS lesions. ALE as a complication requires long-term hospitalization and severely impairs an individual's activities of daily living (ADLs) and quality of life (QOL). As a representative case (Figure 1A), an 86-year-old woman with Alzheimer's disease presented at our hospital in 2013 with HZ skin rashes on the trigeminal nerve area, altered consciousness, and seizures in the left half-body. Her CSF was positive in a VZV PCR, and MRI diffusion-weighted imaging revealed high-intensity on the medial temporal lobe. EEG showed left-predominant slowing. The patient's cognitive ability was worsening, requiring transfer to a facility (*7*).

An 84-year-old woman presented with HZ skin rashes on the left thoracic spinal Th11–12 site, fever, and impaired consciousness. Her CSF showed increased cells at 106/μL, VZV PCR-positivity was observed,

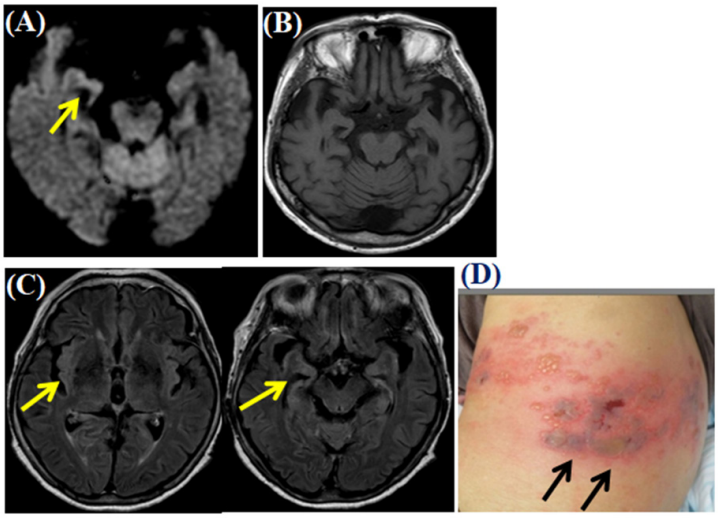

Figure 1. MRI findings of two patients with herpes zoster (HZ) acute limbic encephalitis and HZ skin rash. (A) An 86-year-old female with Alzheimer disease, had acute limbic encephalitis (ALE) with HZ in the first branch of the right trigeminal nerve, and impaired consciousness. This MRI diffusion-weighted image at acute stage showed high-intensity at medial temporal lobe (*arrow only on right side*). **(B)** MRI T1 image at recovery stage exhibited atrophy of the bilateral hippocampal regions. The MRI images are reprinted with permission from reference reported by Matsuoka *et al.* (*7*). **(C, D)** This 84-year-old female with left thoracic spinal Th11–12 HZ showed fever, and impaired consciousness. Contrast-enhanced fluid attenuated inversion recovery (FLAIR) MRI images showed bilateral medial temporal lobe and insular lobe lesions (*arrow only on the right side*).

and MRI exhibited bilateral medial temporal lobe and insula lesions (Figure 1B, C). She received 3 weeks of treatment with acyclovir and prednisolone 3-day pulse and was eventually transferred to a rehabilitation hospital with cognitive disability.

4. Discussion

A typical example of ALE is herpes simplex virus (HSV) encephalitis caused by HSV-1. Acute encephalitis in adults and the elderly caused by HSV-1 appears with fever, impaired consciousness, convulsions, abnormal behavior, hallucinations, and meningeal irritation symptoms; CSF tests show increased cell counts, HSV PCR is positive, and MRI shows limbic system lesions in the hippocampus, amygdala, insula, and/or temporal lobes with a unilateral predominance. An early administration of acyclovir allows approx. half of the treated patients to return to society (*8*). Additional forms of ALE are non-herpetic ALE, N-methyl-D-aspartate (NMDAR) ALE, autoimmune limbic encephalitis, and several paraneoplastic ALE (*9*).

As another herpesvirus group, human herpesvirus-6 (HHV-6-A) has caused ALE in hematopoietic stem cell transplants (*10*); it peaked 3 weeks after transplantation and occurred most frequently 2-6 weeks post-transplant. The hippocampus is the most common site, and recent memory impairment has been observed. The antiviral medication foscarnet was administered. Epstein-Bar virus and cytomegalovirus can also be problematic.

Regarding the HZ ALE literature, in 2001 Tattevin *et al.* reported the first case of subacute limbic encephalitis in an 82-year-old immunocompromised patient with microscopic polyangiitis who presented with a lack of skin rash, altered consciousness, medial temporal lobe lesions on MRI, positive VZV PCR, negative HSV PCR, and a protracted course leading to death on day 43 (*11*). Shindo *et al.* described the case of an 83-year-old woman with Parkinson's disease who presented with somnolence, fever, a painful rash in the left lumbar region, a CSF cell count at 344/μL, positive VZV PCR, negative NMDR antibodies, and symmetrical high-intensity lesions in the limbic system on MRI (*12*).

Among the 105 patients with acute encephalitis treated at our hospital during the years 2002-2012, we observed 20 cases of influenza encephalopathy, 14 of HSV-1 encephalitis, 10 of NMDR limbic encephalitis, and five with VZV acute encephalitis, but no cases of HZ ALE (*13*). Our comparison of these data with the study results from the same facility over the prior 10 years (2012-2023) revealed an increasing trend in HZ ALE cases.

In their study of 96 patients with HSV and VZV CNS infections, Kaewpoowat *et al.* observed 18 patients with VZV CNS infection including five encephalitis patients (*14*). Similarly, in a comparison of HSV1, HSV2, and HZ neurological complications by Lee *et al*., seven patients had HZ encephalitis, but their MRI findings revealed multiple lesions due to vasculitis (*15*). VZV limbic encephalitis was not described in these HZ complication studies.

In our group's paper on HZ neurological complications in the limbs and trunk, we noted that the spread of VZV from latency in the spinal ganglia could take four routes: *via* the spinal roots, ascending the spinal cord, multiple roots, or intrathecal spread (*16*). In patients with HZ ALE, the routes from the cranial ganglia to the limbic areas may be more variable and include pathways

via the brain-stem root, carotid artery, meningeal spread, or viremia. In HSV ALE, the olfactory nerve pathway is considered to be the predominant invasive route of infection (*17,18*), but in HZ ALE, the wide range of routes of VZV seems to be a distinctive characteristic.

The numbers of chickenpox cases have recently decreased due to the widespread use of chickenpox vaccination in children, and the antibody titers in adults and the elderly have decreased more quickly, increasing the risk of HZ in adults and the elderly. Of the present 15 patients with HZ CNS complications, most had no history of prior vaccination. It is expected that vaccination with live chickenpox vaccine or a subunit vaccine will reduce the risk of HZ infection in adults and the elderly (*19,20*).

Our study has limitations to consider; it was a retrospective analysis of patients in a single region. There were no autopsy cases, and thus no autopsy findings to support the pathology in HZ CNS complications were available.

In conclusion, our study of HZ CNS complications identified five patients with HZ ALE whose average age was 82.4 years and whose underlying diseases included hypertension, Alzheimer disease, and hyperlipidemia. The major skin rashes of these five patients were on the trigeminal nerve region, with two cases of generalized rashes. The five patients had a positive CSF VZV PCR result, and MRI showed medial temporal lobe lesions. With the widespread use of varicella vaccines, the risk of developing HZ is increasing in adults and elderly individuals, and attention should be paid to the HZ CNS complication HZ ALE.

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

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Letter

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Primary hepatic angiosarcoma mistaken for a giant hemangioma

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SUMMARY: Primary hepatic angiosarcoma (PHA) is a rare hepatic mesenchymal tumor that accounts for 2% of all primary malignant liver tumors. It typically presents with nonspecific symptoms, is highly aggressive, and there are limited treatment options. Imaging characteristics of PHA overlap with that of hepatic hemangioma, a common benign hepatic lesion, creating a potential diagnostic pitfall. We present a case of PHA that mimicked hepatic hemangioma on imaging. We review the differentiating characteristics between these two hepatic tumors. PHAs demonstrate irregular/infiltrating margins, higher lesion multiplicity, higher risk of tumor rupture, and rapid growth, which are not typically seen with hepatic hemangiomas.

Keywords: primary hepatic angiosarcoma, hepatic hemangioma, magnetic resonance imaging

Primary hepatic angiosarcoma (PHA) is a rare tumor of mesenchymal origin that makes up 2% of all primary malignant liver tumors (*1*). These tumors are more common in men, with a ratio of 3-4:1 male to female. The median age at diagnosis is in the fifth or sixth decade of life (*1-4*). PHAs have been associated with environmental exposure to chemicals like thorotrast, polyvinyl chloride, and arsenic; however, most patients have no known chemical exposures (*5*).

Symptoms at presentation are typically nonspecific and can include abdominal pain, weight loss, weakness, and fatigue (*1,2*). Up to 27% of patients present with spontaneous tumor rupture and intraperitoneal hemorrhage (2). Many patients present with multiple liver masses and metastatic disease (*1,3*).

Computed tomography imaging characteristics of PHA as described in the literature are highly variable. These masses are generally hypoattenuating to liver parenchyma on both arterial and portal-venous phase. Some lesions are hyperattenuating on arterial phase imaging and isoattenuating on portal-venous phase imaging. Tumours can show fluid-fluid levels, which are postulated to be from intra-tumoral hemorrhage (*6*). Some PHA masses can show the characteristic peripheral arterial enhancement typically associated with hemangiomas (*2,6,7*).

On magnetic resonance imaging (MR), PHA has irregular areas of high signal intensity on T1-weighted imaging, suggesting hemorrhage (*8*). There can also be fluid-fluid levels on T2-weighted imaging, with heterogenous architecture and focal areas of high intensity with septum-like or rounded areas of low intensity on T2 sequences. There is often heterogenous enhancement on arterial and portal-venous phases with progressive delayed enhancement. There can also be high inter-lesional variability on diffusion weighted imaging (*5*).

Pathologically, PHAs demonstrate atypical large pleomorphic sinusoidal cells that infiltrate and spread along vascular channels, replacing normal endothelial cells. Polynuclear giant cells may also be seen. PHAs can invade portal or hepatic vein branches (*1*).

Unfortunately, treatment options are limited with median survival without treatment ranging from 5-7 months (*1,2,9*). Hepatic resection has been associated with increased overall survival but is confounded by the fact that patient eligibility for resection implies earlier stage disease (*9*). Liver transplant is not a viable option due to high tumor recurrence rate (*2*). PHAs are also radioresistant. Chemotherapy is typically palliative, without a standardized regimen.

A 70-year-old woman presented to the emergency department with right upper quadrant pain. She had no nausea, vomiting, diarrhea, or hematochezia. Initial laboratory studies including blood counts, electrolytes, renal function, bilirubin, and liver enzymes were normal. Abdominal ultrasound revealed a heterogenous, hypervascular, and predominantly solid mass in the right lobe of the liver measuring 9 × 6 cm. Other smaller echogenic lesions were seen scattered around the liver.

Further investigation with abdominal MR was

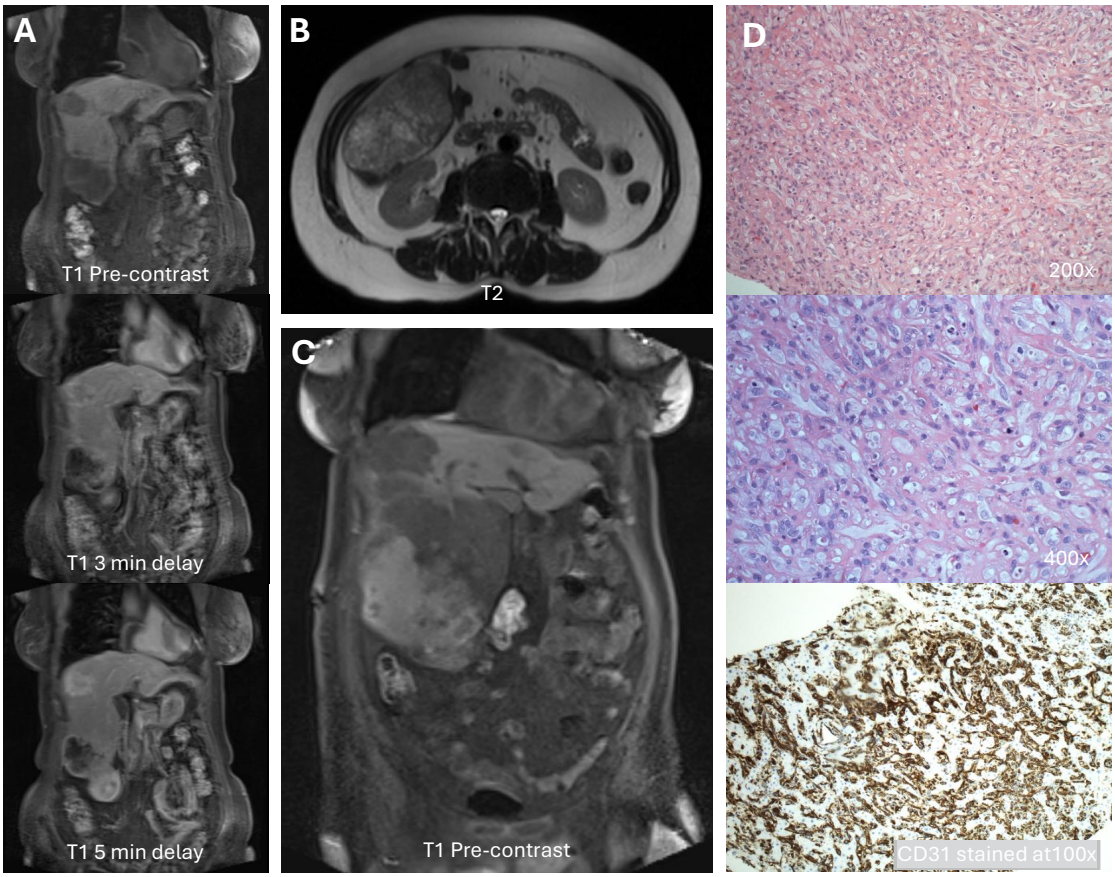

Figure 1. MR and pathological characteristics of hepatic angiosarcoma. (A) T1 weighted pre-gadolinium contrast and 3-minute and 5-minute delayed post-contrast coronal images of the liver. Hepatic masses are low signal on pre-contrast images. They demonstrate nodular gradual peripheral enhancement on post-contrast images. **(B)** T2 weighted axial image of the liver showing the heterogeneously hyperintense mass, not characteristic for hemangiomas. **(C)** T1 weighted pre-gadolinium contrast coronal image of the liver taken five months after images in "A" demonstrating marked enlargement of the hepatic masses. **(D)** The sections show proliferation of epithelioid malignant endothelial cells replacing the normal sinusoid lining endothelial cells. The tumor cells are large, have mostly wider spindle to oval contours with associated nuclear atypia. The tumor cells show positive strong expression of CD31 supporting their endothelial source.

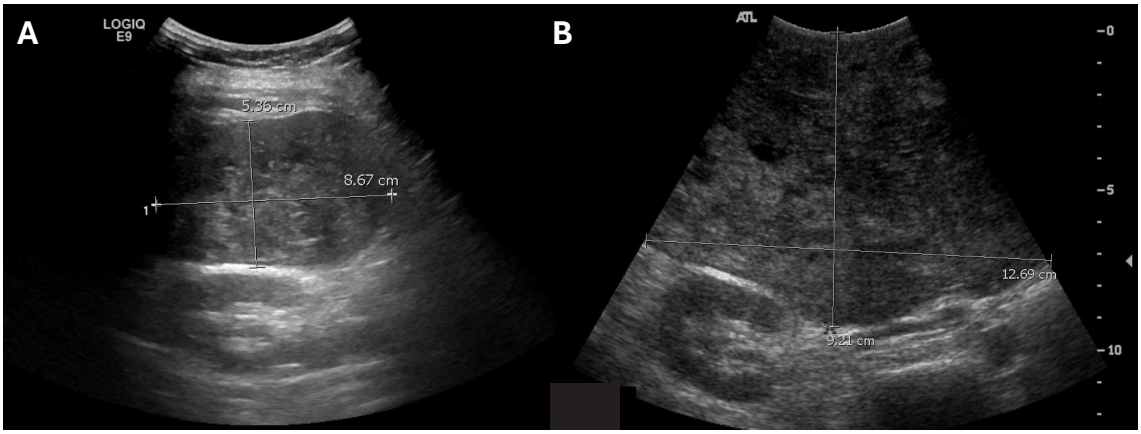

Figure 2. Initial (A) and intraoperative (B) ultrasound images of the large right hepatic mass. The mass is heterogenous and rapidly enlarged in the span of five months.

pursued. The MR demonstrated a heterogenous lesion measuring 7 × 7 × 8 cm with T1 hypo- and T2 hyperintense signal. Gadolinium enhanced images showed nodular peripheral enhancement with partial centripetal fill-in in the dominant mass – characteristic of a hemangioma, which was the initial diagnosis. The other smaller hepatic lesions initially seen on ultrasound demonstrated similar imaging characteristics (Figure 1, A and B).

Because the patient had persistent symptoms,

surgical resection of this lesion was attempted five months after initial presentation. In the operating room, the mass was much larger than described on preoperative imaging, confirmed with intraoperative ultrasound (Figure 2). There were also characteristics of malignancy, with evidence of omental and transverse colon invasion. The resection was aborted because the patient had a small left hepatic lobe and would not have been able to tolerate an extended right hepatic lobectomy. Intraoperative biopsies of the lesion were suspicious for hepatocellular carcinoma or angiosarcoma.

Final pathology analysis of the biopsies revealed proliferation of epithelioid malignant endothelial cells dilating the sinusoids and compressing hepatocytes. There was prominent nuclear atypia and mitotic activity. Immunohistochemistry is positive for CD31 and CD34 – endothelial markers, in keeping with angiosarcoma (Figure 1D).

Follow-up abdominal MR two days after the aborted surgery re-demonstrated a markedly enlarged tumor, now measuring 15 × 10 × 10 cm again with lobular peripheral enhancement with some centripetal fill in on delayed enhancement (Figure 1C). The other smaller hepatic lesions were also enlarged. There was new evidence of splenic and pulmonary metastases. The patient decided to move out of the country and therefore further follow-up was not possible.

The rarity of PHAs combined with initially similar imaging characteristics to hemangiomas, a more common hepatic lesion, creates a potential diagnostic pitfall of which radiologists need to be aware (*7*). In our case, the PHA did show peripheral nodular arterial enhancement with centripetal progression on delayed imaging and an enhancement intensity that followed blood pool, findings that overlap with hemangioma. However, further evaluation of the initial MR showed heterogeneous intermediate to high T2 signal intensity in the larger lesions with irregular/infiltrative margins, a feature not typical for hemangiomas, which show uniform high T2 signal intensity and smooth margins. Other characteristics that can differentiate PHA and hemangiomas include lesion multiplicity, tumor rupture, rapid growth making it important to compare to previous imaging studies, and metastases such as to the spleen, lungs and lymph nodes. If there is diagnostic uncertainty, short-term imaging follow-up, evaluation with F-18 fluorodeoxyglucose positron emission tomography, or biopsy should be considered.

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IRDR
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