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Intractable & Rare Diseases Research publishes Original Articles, Brief Reports, Reviews, Policy Forum articles, Case Reports, News, and Letters on all aspects of the field of intractable and rare diseases research. All contributions should seek to promote international collaboration.
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Accessibility of drugs for rare diseases in China: Policies and current situation

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Summary

"Poor accessibility to drugs" is the most problematic issue for patients with rare diseases in China. In recent years, China has issued a number of policies, such as prioritizing speeding up the evaluation for rare disease drugs, publishing national rare disease lists and giving priority to treatments for severe diseases like rare diseases during annual adjustments of National Medical Insurance Medicine Catalogue to improve the accessibility of rare disease drugs. From the outcome perspective, the evaluation of rare disease drugs takes 3 months shorter than ordinary drugs, basic research projects have been started and the number of rare disease drugs included in National Medical Insurance Medicine Catalogue has increased to 50. However, the policies’ effects on new drug research and development, rare disease diagnosis and treatment as well as drug pricing are limited. It is recommended to learn the tilt policy of research and development for rare disease drugs from foreign countries and the mechanism of medical insurance funding and patient co-payments. Thus it is important to improve the availability, accessibility and affordability of rare diseases drugs based on the Chinese context.

Keywords: Rare disease, accessibility, orphan drug, policy, medical insurance

1. Introduction

Difficult to diagnose, difficult to treat and difficult to get medications are embarrassing situations for patients with rare diseases not only in China but also for rare disease patients all over the world. Due to previous hospital procurement restrictions, physician prescription restrictions, and outpatient reimbursement restrictions, having no access to drugs is a problem crying for solution for patients with rare diseases in China (1). Generally, accessibility includes availability, which involves research, development and market access of drugs, adaptability, which involves the construction of a diagnosis and treatment system, and affordability, which involves the pricing and inclusion in medical insurance (2-4). Thus, the following discussion about the current situation of accessibility to rare disease drugs in China will be from three perspectives, availability, adaptability and affordability.

2. The current national policies on orphan drugs

China's attention to rare diseases started late. There are no special rare disease laws and policies at the national level, and there is no specific rare disease drug (including orphan drug) policy. However, in recent years, as society's attention to rare diseases has increased, the importance of orphan drugs has gradually appeared in various policy documents (Table 1).

It can be seen from various policies that China has increased the accessibility of orphan drugs and implemented priority evaluation. Especially, the evaluation of rare disease drugs is required to be done in 3 months, which has greatly accelerated the speed of new drug listings. In the importing process, value added tax is levied at 3%. In terms of basic research, the publication of National Rare Disease List involved
Table 1. Current effective national policies about rare diseases

<table>
<thead>
<tr>
<th>Item</th>
<th>Category</th>
<th>Effective time</th>
<th>Policy &amp; Regulation</th>
<th>Content about Rare Diseases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Availability</td>
<td>Development and imitation of drugs</td>
<td>2012 (5)</td>
<td>The Notice of the State Council on National Drug Safety During the 12th Five-Year Plan issued by the State Council.</td>
<td>To encourage the development of orphan drugs and suitable dosage forms for children.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2017 (6)</td>
<td>The Opinions of the State Council on Reform of the System of Evaluation, Review and Approval of Drugs and Medical Devices issued by the General Office of the State Council.</td>
<td>To support the development of drugs and medical devices for rare diseases.</td>
</tr>
<tr>
<td></td>
<td>Registration and approval</td>
<td>2007 (7)</td>
<td>The Measures for the Administration of Drug Registration issued by former China Food and Drug administration.</td>
<td>Special approval for new drugs with obvious clinical efficacy in the treatment of AIDS, malignant tumors, rare diseases etc.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2013 (9)</td>
<td>The Opinions of the China Food and Drug Administration on deepening the reform of evaluation and approval systems and encouraging innovation on drugs issued by former China Food and Drug Administrations.</td>
<td>Prioritize and speed up the evaluation of rare disease drugs.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2015 (10)</td>
<td>The Opinions of the State Council on Reform of the System of Evaluation, Review and Approval of Drugs and Medical Devices issued by the State Council.</td>
<td>Accelerated evaluation and approval of innovative drugs for the prevention and treatment of diseases such as rare diseases, sub-neoplastic diseases, AIDS and major infectious diseases.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2016 (11)</td>
<td>The Notice of the General Office of the State Council on issuing the 2015 Major Task List on Deepening the Medical and Health Care System reform issued by the General Office of the State Council.</td>
<td>Further smooth the special channel for evaluation and approval of rare disease drugs and clinical urgently needed drugs.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2017 (13)</td>
<td>The Policies of the China Food and Drug Administration regarding Encouraging Innovation and Accelerating the Evaluation and Approval Systems on Drugs and Medical Devices (Consultation Paper) issued by the former China Food and Drug Ministration.</td>
<td>Applicants for rare disease treatments and medical devices may apply for clinical trials for reduction and exemption; and rare-drug treatment drugs and medical devices that have been approved for marketing abroad, supplement relevant research within the prescribed time after listing.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2017 (14)</td>
<td>Opinions of the China Food and Drug Administration encouraging innovative implementation of prioritized evaluation and approval on drugs issued by the former China Food and Drug Administration.</td>
<td>Drug registration for rare diseases can be included in the scope of prioritized evaluation and approval.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2018 (15)</td>
<td>Notice of the National Medical Products Administration and the National Health Commission on Optimizing Review and Approval of Registration of Medical Products issued by the National Medical Product Administration and the National Health Commission.</td>
<td>Orphan drugs can submit clinical trial data obtained overseas and directly apply for drug listing registration, which meeting the requirements of the Drug Registration Management Measures and related documents may directly approve the import.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2018 (16)</td>
<td>Interim Measures of the National Medical Products Administration for Protection of Pharmaceutical Test Data (Consultation Paper) issued by the former China Food and Drug Administration.</td>
<td>Orphan drugs are listed as the target of data protection and a 6-year data protection period is granted to them since the indication firstly approved in China.</td>
</tr>
</tbody>
</table>
urgently needed drugs for rare disease treatment were included in the "imperatively needed clinical
drugs that should be researched and developed" and
9 research and development projects were carried out
with a central government's financial budget of more
than RMB 50 million (25). On May 29, 2019, the
Center for Drug Evaluation, National Medical Product
Administration published "The Basic Consideration of
Using Real World Evidence to Support the Research
and Development of Drugs (Consultation Paper)" and
proposed that the clinical trials of rare disease drugs can
use the real world data formed by the natural disease
cohort as an external control to help the research (26).

However, due to the weak foundation of China's drug
research and development, coupled with slow progress
in basic research and no special incentives from the
country (such as internationally-recognized research
and development grants, tax reduction policies,
etc.), the company lacks research and development
incentives. At present, there are no new drugs for rare
diseases coming out in China.

121 diseases, which greatly increased the attention to
rare diseases (18).

3. The current accessibility to rare disease drugs in
China

3.1. Availability of rare disease drugs

3.1.1. The level of research, development and imitation
is still low

In 2012, "the Notice of the State Council on National
Drug Safety During the 12th Five-Year Plan" (5) encouraged research and development of rare disease
drugs and suitable dosages for children. Furthermore,
"the Opinions of the State Council on Reform of the
System of Evaluation, Review and Approval of
Drugs and Medical Devices" (6) encouraged research
and development of pharmaceuticals and medical
devices for the treatment of rare disease. In the 2017
and 2018 new drug project application guidelines,
3.1.2. Registration and approval are speeding up and the number is increasing

In terms of speed, it was proposed that rare disease drugs should be reviewed and evaluated within three months (27). From the quantity perspective, more than 40% of around 400 orphan drugs listed in the US haven’t been applied in China (28). Due to the lack of a unified definition of rare disease in the world and there is no clear definition of rare diseases in China, China Organization for Rare Disorders used the National Rare Disease List as a sample to organize the pharmaceuticals globally listed for the 121 rare diseases from the list (Figure 1) (1). From the result, out of the 121 rare diseases, 47 diseases also have no therapeutic drugs (mainly in the US/EU/Japan). However, there are 79 drugs outside the country but not listed in the country, involving 21 diseases. There are still 35 listed drugs that have no indications from the list.

It is worth noting that in 2018, 13 rare disease drugs involving 10 rare diseases were successfully applied for listing through priority review and approval. There are 20 rare disease drugs involving 12 rare diseases included in the list of Clinically-needed Foreign New Drugs (first batch) (19) issued by Centre for Drug Evaluation, National Medical Products Administration. In 2019, 14 rare disease drugs were included in the list of clinically urgent new drugs (second batch) (20).

3.2. Adaptability of rare disease drugs

3.2.1. Basic research projects started

Although basic research on rare diseases in China is weak (29), with the continuous attention of society to rare diseases, the basic data is constantly improving. In December 2016, the China Research Hospital Association Rare Diseases Branch was established in Beijing. At the same time, the National Key Research and Development Program "Rare Disease Clinical Cohort Study" and "Rare Diseases Precision Diagnosis and Treatment Technology and Clinical Standard Research" project were officially launched (30). In June 2017, the National Key Research and Development Program "Chinese Severe Diseases and Rare Diseases Clinical and Life Omics Database" was launched (31). In the same year, the National Rare Disease Registration System (NRDRS) was officially launched, which would create an information resource platform and a biobank with gene, protein, metabolomics and molecular imaging diagnostic platforms. By the end of 2018, NRDRS had registered more than 100 rare diseases and more than 30,000 cases (32).

3.2.2. The current situation of diagnosis and treatment is not optimistic

Although in recent years, China has made many breakthroughs in the construction of a rare disease diagnosis and treatment system, in 2017, Shanghai edited the first rare disease monograph "Treatable Rare Disease", which provided diagnosis and treatment guidelines for 117 different rare diseases (33). In 2018, the China Alliance of Rare Diseases was established which became the first national and non-profit communication platform for rare diseases (34). In 2019, the Rare Diseases Diagnosis and Treatment Guidelines (2019 Edition) involving 121 rare diseases were released. In the same year, the National Health Commission announced that 324 hospitals with strong diagnosis and treatment ability as well as relatively more cases would be selected to establish a rare disease diagnosis and treatment collaboration network (35). However, patients with rare diseases still face the dilemma of having no pharmaceuticals for treatment. Among the more than 7,000 known rare diseases, less
than 10% of the rare diseases have approved therapeutic drugs or interventions. From 2014 to 2018, the Chinese Organization for Rare Disorders surveyed 5,810 patients with rare diseases (36). The results showed that 42% of patients did not receive any treatment, and most of the patients who received treatment failed to take a sufficient amount of medicine in a timely way. In the National Rare Disease List, only 53 rare diseases have therapeutic drugs listed, and 43 kinds of drugs involving 33 rare diseases have been listed in China but have not registered corresponding rare disease indications, which indirectly leads to clinicians making over-inflated prescriptions. But this undoubtedly brings great risk of medication. Finally, the ”last mile” of rare disease drugs is still full of challenges, such as bidding and purchasing, hospital purchase list, prescription restrictions, outpatient reimbursement, and restrictions on designated medical institutions as well as pharmacies.

3.3. Affordability of rare disease drugs

3.3.1. Pricing policy needs to be improved

Since June 2015, China has eliminated the way in which the government manages the price of medicines in a unified manner. The price of different types of drugs will be set by different methods. For rare diseases drugs, there are the following types: those included in the medical insurance list, the National Healthcare Security Administration formulates the medical insurance payment standard; for drugs with patents and exclusive production, the price is determined by multiple parties to negotiate and set the price; for other drugs, the enterprises will mainly price them independently. Because the current domestic rare disease drugs are mainly imported from abroad, most of them are patented drugs or exclusive products, which leads to a high price. For this reason, the State Council issued "the Notice of the Customs Tariff Commission of the State Council on the Provisional Import and Export Tariff Rate and Other Tariff Rate Adjustment Plan for 2019" (37), involving some raw materials of rare disease drugs to implement zero tariff. In 2019, the Ministry of Finance issued the "Notice by the Ministry of Finance, the General Administration of Customs, the State Administration of Taxation and the National Medical Products Administration of the VAT Policies on Drugs for Rare Diseases" (38), the value added tax of 21 rare diseases and 4 active pharmaceutical ingredients were reduced. Despite this, high-priced drugs still face difficulties entering medical insurance list, while low-cost drugs face a crisis of being discontinued or even having production stopped.

3.3.2. Number of rare disease drugs continuously increases

In 2017, China first introduced two orphan drugs into the medical insurance catalogue through national
negotiations (39), and achieved a certain breakthrough in market access for orphan drugs. By the end of 2018, out of 121 rare diseases, 50 drugs for rare diseases had been included in medical insurance, of which 17 were classified as Class A medical insurance (Drugs in Class A medical insurance are fully reimbursed) and 33 were classified as Class B medical insurance (Drugs in Class B medical insurance are partly reimbursed. The percentage depends on the local policies and the type of drugs).

Some provinces and cities in China have guaranteed orphan drugs excluded from the national medical insurance list, but this exploration is still limited to a small number of provinces and cities and a small number of rare diseases/drugs (Table 2).

4. Discussion and Suggestions

Despite the fact that rare diseases have received much attention and favorable policies have been introduced in China, drugs for rare diseases have been imported, but they are mainly concentrated in the drug registration and approval process, and other processes are still weak (Figure 2).

4.1. Formulate a tilt policy for orphan drug research and development

From the perspective of research and development of orphan drugs, although China has introduced relevant incentive policies, it lacks substantial preferential measures, resulting in a lack of research and development incentives. At present, most of the rare diseases in the world lack effective treatment, and there are a large number of blank areas that need to be filled, which leaves room for Chinese biomedical innovation. Therefore, it is recommended to learn
Sharing. The second is to include rare disease drugs in medical insurance for rare diseases, encourage social security model. First, set up a special fund within medical insurance covered and multi-party payment from local experience and establish a government-led, multi-funding and risk sharing. The second is to include rare disease drugs in medical insurance in batches, and to ensure that the rare drugs with the exact effect are preferentially included in the medical insurance. Finally, health technology assessment methods should be introduced to establish a special evaluation process for orphan drugs (50).

In conclusion, rare diseases are not just medical problems, but also social problems. How to promote innovation in the pharmaceutical industry? How to ensure the equity, equality and efficiency and how to solve the sustainability of funds? Despite the valuable experience of other countries and regions, and the exploration of domestic success in some provinces and cities, how to secure the accessibility of rare disease drugs based on the Chinese context still needs much effort.

Acknowledgements

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References


4.2. Establish special drug classification and approval

In the drug registration and approval process, China has accelerated registration through priority evaluation and accelerated approval, which has greatly facilitated the introduction of orphan drugs abroad. It is recommended to give priority to the evaluation and approval of drugs with clear diagnosis and treatable rare diseases. Learn from the experience of introducing抗癌 drugs, further reduce preferential tariffs and VAT. Establish special approval channels for some blood products related drugs to secure rare disease patients, which rely on blood products for intervention or treatment.

4.3. Implement dynamic adjustment to the National Rare Disease List and construct a centers of excellence

Basic research work and diagnosis and treatment capacity construct can directly affect the accessibility of rare diseases drugs. At present, a large-scale clinical cohort study and registration system has been established at the national level (49), but the number of rare diseases is numerous. Therefore, it is recommended to further strengthen the previous epidemiological research and related basic research, implement the dynamic update of the rare disease list. Strengthen the construction of diagnosis and treatment ability, and gradually establish the state and provincial center of excellence to further empower medical staffs to identify, diagnose, and treat rare diseases.

4.4. Try implementation of multi-party co-payment security mode

The cost of rare disease drugs is extremely high. With the main policy of medical insurance in China, how to reduce the burden of patients' drug costs is an important issue to be urgently solved. It is recommended to learn from local experience and establish a government-led, medical insurance covered and multi-party payment security model. First, set up a special fund within medical insurance for rare diseases, encourage social forces to participate through multi-funding and risk sharing. The second is to include rare disease drugs in medical insurance in batches, and to ensure that the rare drugs with the exact effect are preferentially included in the medical insurance. Finally, health technology assessment methods should be introduced to establish a special evaluation process for orphan drugs (50).

In conclusion, rare diseases are not just medical problems, but also social problems. How to promote innovation in the pharmaceutical industry? How to ensure the equity, equality and efficiency and how to solve the sustainability of funds? Despite the valuable experience of other countries and regions, and the exploration of domestic success in some provinces and cities, how to secure the accessibility of rare disease drugs based on the Chinese context still needs much effort.

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Incidence and prevalence of 121 rare diseases in China: Current status and challenges

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Summary

In order to ascertain the current status of and challenges posed by the incidence and prevalence of rare diseases in China, this study teases out data on the incidence and prevalence of 121 rare diseases listed in China's First List of Rare Disease to provide rationales and references for the development and promotion of rare-disease-related policies. The National Health Commission of the People's Republic of China issued the Rare Disease Diagnosis and Treatment Guide (2019) (denoted here as China's Rare Disease Diagnosis and Treatment Guide), which cited data on the incidence/prevalence of 21 rare diseases (21 of 121 rare diseases, 17.36%). Data on 68 diseases (56.20%) were found in monographs, literature databases, and official websites. Data on the incidence/prevalence of 70 diseases were compiled, though no data were available for the 51 remaining diseases. There are published data on the incidence/prevalence of only 14 diseases at the national level. Sources of data on the incidence and prevalence of rare diseases mainly include cases counts from hospitals (40.56%), other sources of data (24.48%), screening (20.98%), cross-sectional studies (8.39%), and estimates from models (7.69%). Data on the incidence/prevalence of rare diseases in China are limited and typically lack accuracy, uniformity, and timeliness. Epidemiological data at the national level are greatly lacking, and data are not amenable to comparison. China recently initiated epidemiological studies of rare diseases at the national and regional level. The country will continue to promote, use, and update its list of common rare diseases, actively encourage the coding and registration of cases of rare diseases, and take actions to collect, share, and use that information.

Keywords: Rare disease, incidence, prevalence, China's Rare Disease Diagnosis and Treatment Guide

1. Introduction

Epidemiological data could indicate the incidence of and changes in rare diseases and lay the foundation for estimation of the rare disease burden for the country or regions, guide the development of orphan drugs, and help with the formulation of rare-disease-related health policies. From the perspective of national health care and social service provision, the population with rare diseases needs to be determined in accordance with their national health care needs and socioeconomic status. The burden of rare diseases is hard to determine because of the difficulty in diagnosis, misclassification, and the lack of appropriate coding; these issues pose major problems with the development of a rare disease health plan. As China works to provide social security to address rare diseases, its top priorities are to enhance the collection
of epidemiological data, to provide health care, and to register patients.

There is no clear standard for recognition of rare diseases in China due to the large population, the lack of epidemiological data on rare diseases, the variety of rare diseases, and other factors. To enhance the management of rare diseases in China and improve the diagnosis and treatment of rare diseases, to protect the rights of patients with rare diseases, and to provide a reference for policymaking by relevant departments, the National Health Commission of the People's Republic of China, the Ministry of Science and Technology, the Ministry of Industry and Information Technology, the National Medical Products Administration, and the National Administration of Traditional Chinese Medicine issued China's First List of Rare Disease in May 2018 (denoted here as China's Rare Disease List (1,2)). In February 2019, the National Health Commission issued the Rare Disease Diagnosis and Treatment Guide (2019) (denoted here as China's Rare Disease Diagnosis and Treatment Guide), which cited data on the incidence/prevalence of rare diseases based on China's Rare Disease List (3).

The current study is based on China's Rare Disease Diagnosis and Treatment Guide and has searched monographs, literature databases and official websites in the field of rare diseases, such as Treatable Rare Diseases edited by Chen et al., the Compendium of China's First List of Rare Diseases edited by Zhang et al., the CNKI database, the Wanfang database, and the official website of the Taiwan Health Promotion Administration. Data on the incidence/prevalence of 121 rare diseases in China's First List of Rare Diseases have been compiled and the current status of and challenges posed by the incidence/prevalence of rare diseases in China have been ascertained in order to provide references for the formulation of rare-disease-related policies.

2. Current incidence/prevalence of 121 rare diseases in China

China's Rare Disease Diagnosis and Treatment Guide cites the incidence/prevalence of 21 rare diseases (21 of 121 rare diseases, 17.36%). Data on 68 diseases (56.20%) were retrieved from the monographs, literature databases and official websites. Data on 70 (57.85%) diseases were compiled, though no data were available for the remaining 51 diseases (42.15%). There are data on the incidence/prevalence of only 14 diseases out of 70 at the national level; data on the other 56 diseases are regional. Details are shown in Table 1. The sources of data on 70 diseases can be divided into 5 categories: cross-sectional studies (national level, regional level), screening (newborn screening and other screening), cases seen at hospitals, estimates from models, and other sources of data. Table 2 shows that data on 70 rare diseases have come from 143 sources, the top 3 of which are cases seen at hospitals (40.56%), other sources of data (24.48%), and screening (20.98%). The sources of data for China's Rare Disease Diagnosis and Treatment Guide are mostly from other sources of data (13.29%) and newborn screening (6.99%). An additional source of data is the number of cases reported to the Taiwan Health Promotion Administration (37.06%).

Newborn screening in China started in the 1980s, and it has developed quickly to cover more diseases with the consecutive issuance of related policies, laws, and supporting documents (27). The 20th Anniversary of the Newborn Screening Study Group and the 2018 Newborn Screening Progress Summit were held in November 2018 in Shanghai. At the summit, Professor Zhao of the Children's Hospital, Zhejiang University School of Medicine, said in a special report on "The progress of China's newborn screening" that China's newborn screening covered 97.5% of the country and that tandem mass spectrometry was used in some regions to detect various rare disease (28). Newborn screening helps to provide data on the incidence/prevalence of rare diseases. For example, the prevalence of hyperphenylalaninemia is 9.62/100,000 persons (3), which is calculated based on screening data from 35 million newborns from 1985 to 2011. Based on 17.96 million pieces of data collected by the Newborn Screening Study Group of the Chinese Preventive Medicine Association, the incidence of phenylketonuria is 8.5/100,000 in China (12).

Taiwan established a rare disease reporting system including rare disease incidence, treatment fees, and treatment outcomes in 2000. It also issued the "Regulations on Implementation of Taiwan's Rare Disease and Orphan Drug Act" in 2000 and it stipulated that medical professionals should report any patients with rare diseases or anyone who died from a rare disease to relevant government departments in Taiwan. A disease is deemed to be a rare disease when its incidence is less than 0.01%, it is recognized by the Rare Disease and Drug Review Committee, and it is documented by a relevant department in Taiwan. Only recognized rare diseases are covered by health insurance, which greatly encourages patients to apply for certification and doctors to report the disease. This also guarantees the smooth running of the rare disease reporting system (29). The Taiwan Health Promotion Administration updates the number of reported cases every month, and the prevalence of rare diseases is calculated based on statistics prior to December 31, 2018 (9) and the population of Taiwan (30). Table 2 shows that 53 rare diseases on China's Rare Diseases List were reported in Taiwan.

3. Challenges posed by the incidence/prevalence of rare diseases: Lack of baseline data and comparability within data

Data on the incidence/prevalence of 70 (57.85%) of 121 rare diseases in China's First List of Rare Diseases
Table 1. Incidence and prevalence of rare diseases in China’s First List of Rare Diseases

<table>
<thead>
<tr>
<th>No.</th>
<th>Rare disease</th>
<th>National Diagnosis and Treatment Guide published data (1)</th>
<th>Other published data</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Incidence/100,000 persons</td>
<td>Prevalence/100,000 persons</td>
</tr>
<tr>
<td>1</td>
<td>21-hydroxylase deficiency</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>2</td>
<td>Albinism</td>
<td>5.56</td>
<td>/</td>
</tr>
<tr>
<td>3</td>
<td>Alport syndrome</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>4</td>
<td>Amyotrophic lateral sclerosis</td>
<td>0.6 (Hong Kong)</td>
<td>3.1 (Hong Kong)</td>
</tr>
<tr>
<td>5</td>
<td>Angelman syndrome</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>6</td>
<td>Arginase deficiency</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>7</td>
<td>Asphyxiating thoracic dystrophy</td>
<td>/</td>
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<td>13</td>
<td>Biotinidase deficiency</td>
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<td>15</td>
<td>Primary carnitine deficiency</td>
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<td>2.4 (Shanghai)</td>
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<td></td>
<td>3.1 (Zhejiang)</td>
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<td>0.8 (Taiwan)</td>
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<td>16</td>
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<td>17</td>
<td>Charcot-Marie-Tooth disease</td>
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<td>24</td>
<td>Coronary artery ectasia</td>
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<td>Diamond-Blackfan anemia</td>
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<td>Erdheim-Chester disease</td>
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<td>Fabry disease</td>
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<td>Familial Mediterranean fever</td>
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<td>Fanconi anemia</td>
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<td>Galactosmia</td>
<td>/</td>
<td>0.53 (Zhejiang)</td>
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<td>31</td>
<td>Gaucher disease</td>
<td>1.24 (Shanghai)</td>
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<td>Generalized myastenia gravis</td>
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<td>33</td>
<td>Gitelman syndrome</td>
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<td>34</td>
<td>Glutaric acidemia type I</td>
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<tr>
<td>35</td>
<td>Glycogen storage disease (type I, II)</td>
<td>2 (Taiwan)</td>
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Note: Unless otherwise indicated, data are national data from China.
Table 1. Incidence and prevalence of rare diseases in China's First List of Rare Diseases (continued)

<table>
<thead>
<tr>
<th>No.</th>
<th>Rare disease</th>
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<th>Other published data</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>Incidence/100,000 persons</td>
<td>Prevalence/100,000 persons</td>
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<td>20 (Male, Blood group A); 4 (Male, Blood group B)</td>
<td>2.73 (China mainland)</td>
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<td></td>
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<td>37</td>
<td>Hepatolenticular degeneration</td>
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<tr>
<td>38</td>
<td>Hereditary angioedema</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>39</td>
<td>Hereditary epidermolysis bullosa</td>
<td>/</td>
<td>/</td>
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<td>40</td>
<td>Hereditary fructose intolerance</td>
<td>/</td>
<td>/</td>
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<td>41</td>
<td>Hereditary hypomagnesemia</td>
<td>/</td>
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<td>42</td>
<td>Hereditary multi-infarct dementia</td>
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<tr>
<td>43</td>
<td>Hereditary spastic paraplegia</td>
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<td>44</td>
<td>Holocarboxylase synthetase deficiency</td>
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<td>45</td>
<td>Hyperhomocysteinemia</td>
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<td>/</td>
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<tr>
<td>46</td>
<td>Hyomozygous familial hypercholesterolemia</td>
<td>/</td>
<td>/</td>
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<tr>
<td>47</td>
<td>Huntington's disease</td>
<td>/</td>
<td>/</td>
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<td>48</td>
<td>Hyperornithinemia-hyperammonemia-homocitrullinuria syndrome (HHIHS)</td>
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<tr>
<td>49</td>
<td>Hyperphenylalaninemia</td>
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<td>9.62</td>
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<td>50</td>
<td>Hypophosphatasia</td>
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<td>51</td>
<td>Hypophosphatemic rickets</td>
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<td>52</td>
<td>Idiopathic cardiomyopathy</td>
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<td>Idiopathic hypogonadotropic hypogonadism</td>
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<td>54</td>
<td>Idiopathic pulmonary arterial hypertension</td>
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<td>Idiopathic pulmonary fibrosis</td>
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<td>56</td>
<td>IgG4 related disease</td>
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<td>57</td>
<td>Inborn errors of bile acid synthesis</td>
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<td>58</td>
<td>Isovaleric acidemia</td>
<td>0.63</td>
<td>0.63 (Shanghai) (12)</td>
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<td>/</td>
<td>0.27 (Taiwan) (12)</td>
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<tr>
<td>59</td>
<td>Kallmann syndrome</td>
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<td>60</td>
<td>Langerhans cell histiocytosis</td>
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<td>61</td>
<td>Laron syndrome</td>
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<td>62</td>
<td>Lever hereditary optic neuropathy</td>
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<td>63</td>
<td>Long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency</td>
<td>0.40</td>
<td>≥ 1.092 (Xingtai, Hebei)</td>
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<td>64</td>
<td>Lymphangioleiomyomatosis</td>
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<td>66</td>
<td>Lysosomal acid lipase deficiency</td>
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<td>67</td>
<td>Maple syrup urine disease</td>
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<td>0.72 (China mainland)</td>
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<td>1 (Taiwan)</td>
<td>0.56 (Zhejiang) (12)</td>
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<td>68</td>
<td>Marfan syndrome</td>
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Note: Unless otherwise indicated, data are national data from China.
<table>
<thead>
<tr>
<th>No.</th>
<th>Rare disease</th>
<th>Incidence/100,000 persons</th>
<th>Prevalence/100,000 persons</th>
<th>Incidence/100,000 persons</th>
<th>Prevalence/100,000 persons</th>
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<td>McCune-Albright syndrome</td>
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<td>70</td>
<td>Medium-chain acyl-CoA dehydrogenase deficiency</td>
<td></td>
<td>0.66</td>
<td></td>
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<td>71</td>
<td>Methylmalonic acidemia</td>
<td>10 (North China)</td>
<td>3.57 (China mainland)</td>
<td>1.27 (Yancheng, Jiangsu)</td>
<td>3 (Shanghai)</td>
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<td>72</td>
<td>Mitochondrial encephalomyopathy</td>
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<td>73</td>
<td>Mucopolysaccharidosis</td>
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<td>74</td>
<td>Multifocal motor neuropathy</td>
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<td>75</td>
<td>Multi acyl-CoA dehydrogenase deficiency</td>
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<tr>
<td>76</td>
<td>Sclerosis multiple</td>
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<td>Myotonic dystrophy</td>
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<td>79</td>
<td>N-acetylglutamate synthase deficiency</td>
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<td>80</td>
<td>Neonatal diabetes mellitus</td>
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<td>81</td>
<td>Optical neuritis</td>
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<td>Niemann-Pick disease</td>
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<td>83</td>
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<td>Noonan syndrome</td>
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<td>Ornithine transcarbamylase deficiency</td>
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<td>Osteogenesis imperfecta</td>
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<td>87</td>
<td>Parkinson's disease (early-onset; young-onset)</td>
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<td>88</td>
<td>Paroxysmal nocturnal hemoglobinuria</td>
<td>2.7 (Mudanjiang, Heilongjiang)</td>
<td>0.21 (13)</td>
<td>0.063 (6 provinces in China)</td>
<td>0.45 (Taiwan)</td>
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<td>Peutz-Jeghers syndrome</td>
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<td>90</td>
<td>Phenylketonuria</td>
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<td>8.5 (12)</td>
<td>9.62 (12)</td>
<td>1.17 (Taiwan)</td>
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<td>91</td>
<td>POEMS syndrome</td>
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<td>Porphyrin</td>
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<td>93</td>
<td>Prader-Willi syndrome</td>
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<tr>
<td>94</td>
<td>Primary combined immunodeficiency</td>
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<tr>
<td>95</td>
<td>Primary hereditary dystonia</td>
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<td>96</td>
<td>Primary light chain amyloidosis</td>
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**Note:** Unless otherwise indicated, data are national data from China.
### Table 1. Incidence and prevalence of rare diseases in China’s First List of Rare Diseases (continued)

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<th>Other published data</th>
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<td>Prevalence/100,000 persons</td>
<td>Incidence/100,000 persons</td>
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<td>Progressive muscular dystrophy</td>
<td>25.95</td>
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<td>Propionic acidemia</td>
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<td>0.6-0.7</td>
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<td>100</td>
<td>Pulmonary alveolar proteinosis</td>
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<td>101</td>
<td>Pulmonary cystic fibrosis</td>
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<td>102</td>
<td>Retinitis pigmentosa</td>
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<td>103</td>
<td>Retinoblastoma</td>
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<td>104</td>
<td>Severe congenital neutropenia</td>
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<td>Severe myoclonic epilepsy in infancy</td>
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<td>Sickle cell disease</td>
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<td>Silver-Russell syndrome</td>
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<td>Sjögren’s syndrome</td>
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<td>Spinal bulbar muscular atrophy</td>
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<td>Spinocerebellar ataxia</td>
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<td>Tetrahydrobiopterin deficiency</td>
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<td>Tuberous sclerosis complex</td>
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<td>Very long-chain acyl-CoA dehydrogenase deficiency</td>
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<td>117</td>
<td>Williams syndrome</td>
<td>4.26 (Hong Kong)</td>
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<td>Wiskott-Aldrich syndrome</td>
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<td>X-linked agammaglobulinemia</td>
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<td>121</td>
<td>X-linked lymphoproliferative disease</td>
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*Note: Unless otherwise indicated, data are national data from China.*

### Table 2. Sources of data on the incidence/prevalence of 70 rare diseases in China

<table>
<thead>
<tr>
<th>Source of data</th>
<th>Number of diseases (n = 21)</th>
<th>Sources of detailed data [No. (%)]</th>
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<tr>
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<td>Cross-sectional study</td>
<td>Screening</td>
</tr>
<tr>
<td></td>
<td>National level</td>
<td>Regional level</td>
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<tr>
<td>In guidelines</td>
<td>21 (17.36)</td>
<td>1 (0.70)</td>
</tr>
<tr>
<td>Not in guidelines</td>
<td>68 (56.20)</td>
<td>0 (0.00)</td>
</tr>
<tr>
<td>Total</td>
<td>70 (57.85)</td>
<td>1 (0.70)</td>
</tr>
</tbody>
</table>

*Note: Other sources of data mainly include systematic reviews, meta-analyses, and expert opinions.*
have been compiled, though no data are available on the incidence/prevalence of the 51 remaining diseases (42.15%). There are no available data on 107 diseases (88.43%) at the national level. Baseline data on the incidence/prevalence of rare diseases in China are lacking, and some data are not updated in a timely manner. For instance, data on the prevalence on hemophilia in China cited in China's Rare Disease Diagnosis and Treatment Guide were calculated based on survey data from 1986 to 1989. However, the prevalence of hemophilia varies differently in different countries and even in the same country at different times because of economic and other factors. Clearly, the data cited in China's Rare Disease Diagnosis and Treatment Guide do not accurately represent the current prevalence of hemophilia in China (3).

In addition, the sources of data are disparate. Few data come from cross-sectional studies (8.39%); only one source of data was a national cross-sectional study. Most data are from other sources (24.48%). Data in the form of expert opinions are subjective, which will lead to the rather large differences in the incidence/prevalence of a disease and a lack of comparability. In China's Rare Disease Diagnosis and Treatment Guide, an expert estimate of the incidence of paroxysmal nocturnal hemoglobinuria is 1/100,000. However, the estimated incidence is 0.21/100,000 in the Compendium of China's First List of Rare Diseases by Zhang et al. (13) while it is only 0.041/100,000 (25) according to Wang based on the number of reported cases of rare diseases in the China Biological & Medical Literature Database and the population size according to the Sixth National Census. The prevalence of methylmalonic acidemia in Taiwan is 1.16/100,000 (3) according to China's Rare Disease Diagnosis and Treatment Guide, but it is only 0.22/100,000 based on the number of cases of rare diseases reported to the Taiwan Health Promotion Administration (9.30).

Moreover, the incidence/prevalence of some rare diseases is too high. Patients from across the country visiting noted hospitals for treatment might be one reason for this. Other reasons include obvious regional differences, the sample size, or a limited number of interview respondents. More data need to be collected at the national level. Pan studied 106,305 patients who underwent coronary arteriography at Beijing Fuwai Hospital from January 2009 to May 2014 and found that the prevalence of coronary artery ectasia was 656/100,000 (15). Li et al. conducted a cross-sectional study of 15,000 children in Luohu, Henan and found that prevalence of congenital scoliosis was 202.43/100,000, which is far higher than 65-100/100,000 as indicated by the WHO (31). In general, there are few data on the incidence/prevalence of rare diseases in China, and the data that are available lack accuracy, uniformity, and timeliness. Epidemiological data at the national level are greatly lacking, and data are not amenable to comparison.

4. The development and promotion of a national rare disease information platform: Standardized data collection and information sharing

The issuance of China's First List of Rare Diseases is a milestone in China's efforts to address rare diseases, and it symbolizes the country's commitment to address the social security concerns of patients with rare diseases (1). China has made demonstrable progress in policymaking with regard to rare diseases over the past 2 years, and several policies and laws have addressed scientific research, diagnosis and treatment, drug access, and medical care for rare disease (32). China is also studying rare diseases epidemiologically: In September 2016, a Clinical Cohort Study of Rare Diseases (Project No.: 2016YFC0901500) was finalized as a specialized medical research project under the national plan for R&D in key areas as formulated by the Ministry of Science and Technology; the aim is to have more than 20 research institutes collaborate in establishing the first national rare disease registry and to register over 50,000 patients with more than 50 diseases (33). The project also aims to integrate clinical and biological information and to conduct a large-scale cohort study (34). The National Rare Disease Registry (NRDR) was officially launched in June 2017, and more than 100 rare diseases were registered with the NRDR as of August 2018. As of April 29, 2019, information on 35,374 cases of rare diseases was registered. Case registration as part of the project is 70.75% complete (35).

Some localities have also addressed rare diseases; two examples are Shandong Province and Beijing. Shandong launched a "Project to Study and Attempt to Control Rare Diseases in China (No.: 2013BAI07B00) as part of the National Program to Support Science and Technology under "the Twelfth Five-year Plan" (2011-2015). Shandong conducted a large-scale thorough study of rare diseases and established a clinical database of rare diseases (36). The province also collected data on 10,063 cases of rare diseases and more than 1,000 clinical samples (37). In collaboration with research institutes in Shandong and 6 other provinces, the Shandong Association for the Prevention and Treatment of Rare Diseases conducted an epidemiology study of rare diseases in nearly 100 tertiary hospitals in China. Findings indicated that a total of 40,589 patients with rare diseases (2.27% of all hospitalized patients) were seen by 93 hospitals in the 7 provinces. Hospitals diagnosed 952 diseases, and at least half of them were congenital diseases (38). On March 1, 2017, the Association and the Shandong Health and Family Planning Commission began registering cases of rare diseases in China. The two organizations initially registered 68 diseases and nearly 1,700 cases, and their efforts are expanding (37). The Rare Disease Branch of
the Beijing Medical Association has conducted studies of rare diseases since 2013; using the rare diseases included in European websites related to rare diseases as a template, the Rare Disease Branch has collected and analyzed 404,312 cases from tertiary hospitals in Beijing. As a result, the Rare Disease Branch has identified 1,423 rare diseases (38). In 2014, the Rare Disease Branch conducted a "Study on establishing an ICD-10 coding library for rare diseases in China" (39). Preliminary research by the Rare Disease Branch yielded information on 121 diseases in China's First List of Rare Disease, including the number of inpatients, disease distribution by province/municipality, age group, and the rate of repeated hospitalization at 96 level A tertiary hospitals. Although national epidemiological data are lacking, information on diseases in the database has been data-mined, which is also an effective approach for an epidemiological study (40).

5. Conclusion

Data on the incidence/prevalence of 121 rare diseases in China's First List of Rare Diseases are lacking, and sources of data are disparate. China has made preliminary efforts to examine the epidemiology of rare diseases and it has achieved some success, but the completeness, accuracy, uniformity, and timeliness of data need to be improved further. Close cooperation by every party is needed, and several measures should be taken to advance the epidemiological study of rare diseases. In the future, China will promote the use of a uniform rare disease list in its national health care system and ensure that the list is continuously updated. The coding of rare diseases should be actively promoted to improve their traceability in the national health system. Related measures should be encouraged at the national level, and sources of data like government and health care providers should be used extensively to improve the management of rare diseases. National and local offices for registries should be established for specific rare diseases and groups. In accordance with national laws, the governmental health care system should devise tools or measures and allocate funds for additional research projects (41), promote the collection of data from every valid source (including clinical facilities), and share more information on rare diseases such as their incidence and prevalence. Information on diagnosis and treatment should be made more accessible to the public (42).

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Molecular mechanisms and clinical manifestations of rare genetic disorders associated with type I collagen

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1. Introduction

Type I collagen, as an important structural protein, is classified as one of the major fibrillar collagens (1). It is the most common collagen found in the human body and other vertebrates. Type I collagen is distributed widely, including bones, ligaments, tendons, cartilage, skin, liver, heart valves, cornea, lungs and other connective tissues (2,3). It is synthesized as procollagen precursor, which predominately consists of two identical pro-α1(I) and one proα2(I) chains, encoded by COL1A1 and COL1A2, respectively. The N- and C- terminal propeptides of procollagen are cleavage by N-proteinase and C-proteinase correspondingly, to form the central triple helix structure with Gly-X-Y repeat units. Mutations of COL1A1 and COL1A2 genes are associated with osteogenesis imperfecta, some types of Ehlers-Danlos syndrome, Caffey diseases, and osteogenesis imperfect/Ehlers-Danlos syndrome overlapping diseases. Clinical symptoms caused by different variations can be variable or similar, mild to lethal, and vice versa. We reviewed the relationship between clinical manifestations and type I collagen – related rare genetic disorders and their possible molecular mechanisms for different mutations and disorders.

Keywords: Type I collagen, biosynthesis, osteogenesis imperfecta, Ehlers-Danlos syndrome, Caffey disease, N- and C- propeptide, mutation

Summary

Type I collagen is an important structural protein of bone, skin, tendon, ligament and other connective tissues. It is initially synthesized as a precursor form, procollagen, consisting of two identical pro-α1(I) and one proα2(I) chains, encoded by COL1A1 and COL1A2, respectively. The N- and C- terminal propeptides of procollagen are cleavage by N-proteinase and C-proteinase correspondingly, to form the central triple helix structure with Gly-X-Y repeat units. Mutations of COL1A1 and COL1A2 genes are associated with osteogenesis imperfecta, some types of Ehlers-Danlos syndrome, Caffey diseases, and osteogenesis imperfect/Ehlers-Danlos syndrome overlapping diseases. Clinical symptoms caused by different variations can be variable or similar, mild to lethal, and vice versa. We reviewed the relationship between clinical manifestations and type I collagen – related rare genetic disorders and their possible molecular mechanisms for different mutations and disorders.

Keywords: Type I collagen, biosynthesis, osteogenesis imperfecta, Ehlers-Danlos syndrome, Caffey disease, N- and C- propeptide, mutation

1. Introduction

Type I collagen, as an important structural protein, is classified as one of the major fibrillar collagens (1). It is the most common collagen found in the human body and other vertebrates. Type I collagen is distributed widely, including bones, ligaments, tendons, cartilage, skin, liver, heart valves, cornea, lungs and other connective tissues (2,3). It is synthesized as procollagen precursor, which predominately consists of two identical proα1(I) and one proα2(I) peptide chains, encoded by COL1A1 and COL1A2 gene, respectively. Three α-chains fold into a 300 nm-long triple helix with short nonhelical terminal peptides-telopeptides. The triple helical region of each α-chain has the obligatory Gly-X-Y repeats with glycine in every third position. The amino acids in X and Y position of collagen are often proline and hydroxyproline, respectively (4,5).

The heterotrimer is the dominant form of type I collagen. Homotrimers of three α1(I) chains are identified in fetal tissues, tumors and some fibrotic lesions and this isoform is more resistant to cleavage by collagenases than heterotrimers (6-9). In a recessively homozygous oim mice with G deletion at nucleotide 3983 of COL1A2 gene, and recessively cardiac valvular type Ehlers-Danlos syndrome (cvEDS), collagen molecules exist as homotrimers of three α1(I) in the extracellular matrix and proα2(I) chains are not incorporated into procollagen molecules (10-12).

Mutations in COL1A1 and COL1A2 genes cause Caffey disease (OMIM 114000) (13), the arthrochalasia type Ehlers-Danlos syndrome (aEDS) (OMIM 130060, 617821) (14-16), cvEDS is an autosomal recessive genetic form (OMIM 225320) (12) and autosomal dominant osteogenesis imperfecta (OI) (OMIM 166200, 166210, 259420, 166220), postmenopausal osteoporosis (OMIM 166710) (17). Symptomatic patients with different or similar clinical phenotypes are described...
in different disorders and mutations. Subclinical or asymptomatic individuals with these inherited diseases may express an overlap syndrome, or different penetrance and expressivity in patients with the same pathogenic mutations.

This review summarizes the biosynthesis process of type I collagen and investigates the correlation between phenotype and genotype and mechanisms of rare genetic disorders related to \textit{COL1A1} and \textit{COL1A2}.

2. Biosynthesis of type I collagen

The process of type I collagen biosynthesis undergoes multiple intracellular and extracellular steps (Figure 1) \citep{18}. First, procollagen is synthesized on the ribosome and transported into the rough endoplasmic reticulum (rER), where they undergo a series of post-translational modifications. Hydroxylation of prolyl and lysyl residues are catalyzed by prolyl 4\textsuperscript{a}/3\textsuperscript{a}-hydroxylases (P4H, P3H) and lysyl hydroxylases (LH), respectively \citep{19}. O-linked glycosylation of hydroxylysines is followed by the attachment of galactose and then glucose to hydroxylysine residues. These modifications are processed simultaneously with the synthesis and folding of procollagen chains and occur only within the unfolded region of the chains \citep{20-22}. Unlike most proteins, which begin to fold stepwise from N-terminal end to C-terminal end while the rest of the chain is still being synthesized.

The folding process of procollagen initiates from association of two proα1(I) and one proα2(I) peptide chain within C-propeptide region, then stepwise to N-terminal end of the chain. C-propeptide folds in a zipper-like manner only after chain synthesis is completed \citep{21}. Chain recognition sequences (CRSs) of the C-propeptide is involved in the process of folding. It interacts with the CRSs of a neighboring chain during intracellular trimerization of procollagen to ensure correct homotrimeric and heterotrimeric chain stoichiometry \citep{23}. CRSs is variable and responsible for selective assembly of different types of collagen \citep{23,24}. Meanwhile, protein disulfide isomerase (PDI) catalyzes the formation and rearrangement of intra- and inter-molecular disulfide bonds \citep{25}. There are many other proteins involved in the procollagen assembly. ER chaperones like glucose regulated proteins GRP78 (BiP) and GRP94 (GP96) bind to unfolded and partially folded C-propeptide. Misfolding procollagen leads to sequestration of BiP and GRP94, activates conventional unfolded protein response (UPR) signaling, and leads to ER associated degradation (ERAD) of misfolded protein by proteasomes \citep{26-29}. Heat shock protein 47 (HSP47),

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1}
\caption{Schematic representation in the process of synthesis, processing and assembly of type I collagen \citep{18}.}
\end{figure}
as a collagen-specific molecular chaperone, makes the triplex-helix folding favorable (30). It regulates LH2 activity through association to peptidyl-prolyl cis-trans isomerase (FKBP65) and LH2 to form HSP47-FKBP65-LH2-BiP complex. FKBP65, interacting with HSP47, functions to balance the lysyl hydroxylation. BiP increases affinity of the lysyl hydroxylation complex (31).

After assembly, procollagen is transported into Golgi through endoplasmic reticulum to Golgi intermediate compartment (ERGIC), or vesicular tubular clusters (VRC) in a coat protein complex II (COPII)-dependent process. ER membrane protein TANGO1, acts as the receptor for large cargoes- procollagen and organizer of ER exit sites. It is retained in the ER and exported out of the ER in large COPII –coated carriers, and retrieved back to the ER by retrograde coat, COPI. TANGO1 interacts with the C-terminal RDEL retrieval sequence of HSP47 and is exported from ER to Golgi through interacting proteins cTAGE5 and SEC12 (32-34).

In Golgi, N-propeptide and C-propeptide cleavage have been identified in the secretory pathway inside the cell (35). Nevertheless, a predominant fraction of procollagen is secreted with intact N- and C-propeptide into extracellular matrix (36). Then, N-terminal and C-terminal globular regions of propeptides are removed by a disintegrin and a metalloproteinase with thrombospondin repeats (ADAMTS) and bone morphogenetic protein 1 (BMP1)/Tolloid-like proteinases from procollagen to generate triple-helix fibril monomers named tropocollagen (37). Finally, monomers assemble into macromolecular fibers by covalent crosslinks between certain lysine and hydroxylysine residues (19,38). Fiber assembly is tightly regulated in vivo by a variety of molecules. Disruption in collagen fiber formation, structure and function by mutations leads to different bone pathology.

3. Rare genetic disorders caused by COL1A1 and COL1A2 genes

3.1. Osteogenesis imperfecta

3.1.1. Phenotypic and genotypic characteristics of OI

Osteogenesis imperfecta, also known as brittle bone disease, is a genetic connective tissue disorder with genetic and phenotypic diversity (39,40). Skeletal features for OI are an increased susceptibility to bone fractures and reduced bone density, short stature, scoliosis and skull deformities. Extraskeletal characteristics include hearing and sight impairment, dentinogenesis imperfecta, blue sclera, involvement of connective tissue, and cardiovascular and neurological abnormalities to some extent (Figure 2) (41,42). OI has a broad clinical spectrum, ranging from mild to lethal forms. According to Sillence classification system, OI is divided into four types. Type I is the mildest form, patients with type I OI usually have family history, blue sclera, hearing impairment, near normal stature and...
rarely have dentinogenesis imperfecta. Type II OI is perinatal lethal, clinical characteristics include defective cranial ossification and severe skeletal deformity due to intrauterine rib, long bone fractures, and respiratory failure. Type III OI is the most severe form, affected individuals have triangular faces, frontal bossing, coxa vara, basilar invagination, early onset scoliosis, short stature, multiple fractures and long bone deformities (43,44). Type IV is the moderately severe OI form, with overlapping phenotypes ranging from type I to type III. Generally, affected individuals experience broken fracture, mild short stature, dentinogenesis imperfecta, hearing loss, scleae hue. Nearly 85% OI patients belong to type I to type IV, which is induced by COL1A1 and COL1A2 genes encoding proα1(I) and proα2(I) of type I procollagen in an autosomal-dominant inherited form (45,46). Type VI to XIX are predominated by an autosomal-recessive form, and the number of pathogenic genes are multiple, while the total percentage for OI is no more than 20%, the pathogenic genes for autosomal-recessive and X-linked recessive forms includes CRTAP, LEPRE1, PPIB, BMP1, FKBP10, SERPINH1, SERINF1, WNT1, SP7, SERPINF1, TMEM38B, SPARC, MBTPS2 and TENT5A (39,47-49).

3.1.2. COL1A1 and COL1A2 gene mutations of OI

Generally, there’s a relationship between genotype and phenotype for dominant OI caused by type I collagen genes. The molecular defect for type I OI is a null COL1A1 allele due to frame shifts or premature termination codons (PTCs), resulting in a decrease in the amount of structurally normal type I collagen. Splice site mutation can lead to mis-splicing with subsequent PTCs (50).

Substitutions and deletions in the triple helix region of type I collagen is the main mutation pattern for type II to type IV OI, predominated by substitutions of obligatory Gly-X-Y units. Substitutions of residues in this unit make procollagen folding more difficult, for these mismatches will further destabilize the triple helix. Triple-helix destabilization promotes procollagen misfolding, leads to retention of misfolded procollagen in the ER and HSP47 is unregulated (51). ER stress mediated by these mutations affect these cells producing type I collagen to some extent, resulting in structural and qualitative abnormal type I collagen (52-54). Glycine to serine substitution is most prevalent in all kinds of glycine changes. Meanwhile, Glycine to serine or alanine substitutions cause less damage than those of arginine, glutamic acid, aspartic acid or valine. Triple-helix folding of the later is much delayed. Glycine substitutions located in α1(I) tend to be more severe than the α2(I) chain (55,56). Glycine substitutions at C-terminal of collagen chains are more severe than that of N-terminal (57).

COL1A1 or COL1A2 mutations of OI are predominated by variations in the triple-helix region. OI Mutations in N- and C-propeptide or nearby are rare and commonly patients with these terminal mutations have overlapping clinical characteristics with other syndromes. Mutations in the C-propeptide cleavage The author confirm this is right with high bone mineral density (58,59) and moderate to severe forms of OI are identified (60). For mutations in N-propeptide region, uncleaved N-propeptides will be enrolled into collagen fiber, and hence alter the fiber size, structure and strength (61,62). For individuals who have mutations at these terminal regions of type I collagen, possible symptoms with other syndrome should be considered.

3.2. Type I collagen gene related EDS

3.2.1. Cardiac-valvular type Ehlers-Danlos syndrome

Ehlers-Danlos syndrome is a group of connective tissue disorders with phenotypic and genotypic variability. The modified classification based on Villefranche recognizes 13 different subtypes, most of which are linked to mutations in one of the genes encoding collagen proteins, enzymes for post translational modifications, myomatrix proteins and glycosaminoglycans (63,64). Type I collagen-related EDS are rare, with no more than 50 patients in total (65,66).

Cardiac-valvular type EDS (cvEDS) is characterized by cardiac valvular defects, joint hypermobility (generalized or restricted to small joints) and skin hyperextensibility, with atrophic scars, thin skin, and easy bruising. Minor criteria for diagnosis include inguinal hernia, pectus deformities, joint dislocations, and foot deformities (63,67-69). The absence of pro2(I), and α2(I) are observed in all reported seven cvEDS patients (10,12,70,71). Biallelic COL1A2 mutations lead to nonsense-mediated mRNA decay and loss of function was suggested to be the molecular mechanism of cvEDS (10,71). Homozygous pro2(I) deficiency was reported also in type III OI patient, with recurrent fractures including rib fractures, popcorn deformities of the knee, wormian bone, generalized osteopenia, short stature, and hypermobile fingers. Collagen fibrils were mostly normal except for focal areas which were disorganized in size and shape (72). Patients with homozygous mutation of COL1A2 at intron 46 produced non-functional prote2(I), presenting EDS/OI phenotypes including generalized joint hypermobility and foot deformities, pale blue sclerae and a mild increase in bone fragility (73). In all, deficiency of pro2(I) leads to different diseases and phenotypes and its mechanisms need to be further studied.

3.2.2. The arthrochalasia type Ehlers-Danlos syndrome

The arthrochalasia type of EDS, formerly known as VIIa and VIIb, with pathogenic genes of COL1A1 and
COL1A2, respectively. It is different from other types of EDS by major diagnostic criteria of typical congenital hip dislocation, generalized joint laxity with multiple dislocations/subluxations, and skin hyperextensibility. Minor criteria for diagnosis include tissue fragility, easy bruising, muscle hypotonina, kyphoscoliosis and radiologically mild osteopenia (63,68,74,75).

Generally, COL1A1 and COL1A2 gene mutations for aEDS patients are clustered around the exon/intron 5 and 6 region. Heterozygous in frame deletion of N-telopeptide of either proα1(I) or proα2(I) are a common genetic basis for aEDS. Splice site mutations of intron 5 and 6 of COL1A1 or COL1A2 gene lead to whole or partial skipping of exon 6 (76,77). N-telopeptide links N-propeptide to the triple-helix domain. It contains cleavage site for procollagen-N-proteinase and cross-linking lysyl residue. Thus, deletion of N-telopeptide results in incomplete cleavage of procollagen and defective cross-linking. N-propeptide is incorporated within fibrils to form accumulated pNcollagen (77,78).

Until now, over 30 aEDS have been reported and most mutations are caused by COL1A2 mutation (14,65,66,74,77-87). It seems that aEDS caused by COL1A1 have more severe phenotypes than COL1A2 gene. Collagen fibrils in aEDS patients are more loosely and irregular organized, vary widely in diameter and present with ragged cross-sections in outline. This is extremely severe in patients with EDS VIIa compared to EDS VIIb patients (75).

Patient with exon 5 and exon 6 deletion of COL1A1 presented with bilateral dislocation of the hips, extreme joint hypermobility, femoral fracture and normal skin at birth (81). The 3’ acceptor splice site COL1A1 IVS5 -1G>A results in alternative splicing of exon 6. Patients with this mutation have overlapping phenotypes resembling OI, including multiple fractures, wormian bones, generalized dentinogenesis imperfecta, severe kyphoscoliosis, and relative short stature. The skin was generally lax, redundant, and hyperextensible, but without scarring or bruising (81). Mutation of c.543G>A (p.Met181Ile) in the last nucleotide of exon 6 induced abnormal pre-mRNA splicing without exon 6 (13). One of the probands with this mutation presented with typical aEDS symptoms and a fracture history (86). Patients with EDS VIIb are more prevalent than VIIa type and fractures are rarely documented (88). Clinically, there is some overlap between OI and EDS type VII, especially for EDS type VIIa as mentioned above. In reverse, hypermobility is also seen in OI patients (88), overlapping with aEDS.

3.2.3. OI/EDS

Patients with OI/EDS combined clinical symptoms of OI as well as EDS. OI/EDS mutations are restricted to the region of N-terminal and C-terminal of type I collagen. The N-terminal of type I collagen triple helix region, corresponds to exons 7-14 (89-94). The first 85 amino acids at N-terminal of each chain of type I collagen helix are called the N-anchor, which is vital for proper folding and stability of N-terminal end of the triple helix. Mutations at this region lead to incomplete or delayed N-propeptide cleavage and association of pNcollagen with fibrils (89). OI/EDS patients with this type of mutation usually presented mainly by generalized joint hyperlaxity and skin hyperextensibility, early progressive scoliosis as well as mild to lethal OI symptoms including relatively short stature, blue sclerae, infrequent or frequent bone fracture and different levels of osteopenia (Figure 3) (89-91,93). We found ptosis...
in one individual with family history existed in OI/EDS with the mutation of c.3521C>T (Ala1174Val) in COL1A1; this symptom is also displayed in type XV OI caused by the Wnt1 gene (95-97).

Splice site mutations lead to partial or whole exon skipping similar to aEDS, with atypical or mild form of OI. Mosaic deletion of 9 nucleotides from 3150 to 3158 in the coding region of exon 44 results in a frame deletion of three amino acids was identified in a patient’s cultured fibroblasts. The patient with this mutation has mild clinical EDS and OI symptoms (98). Exon duplication with the addition of 477 amino acids (p.Gly181_Lys657dup) to the triple helical domain of proα2(I) chain leads to relatively mild OI/EDS symptoms (91). Patients with missense mutations of c.563G>A (p.Gly188Asp), c.607G>T (p.Gly203Cys) in COL1A1 and c.326G>A (p.Gly109Asp) in COL1A2 all showed cardiovascular problems, which was not observed in patients with mutations of c.587G>T (p.Gly196Val) in COL1A1 (90). For c.671G>A (p.Gly224Asp) mutation of COL1A1, patients have severe type III OI and moderate EDS, collagen fibrils are organized irregularly, with decreased fibril density and decreased fibril diameter (93).

OI/EDS patients with mutation in C-terminal of type I collagen genes (exon 37 to 51) have broad clinical severity. Substitution of Ala1174Val, Arg1036Cys and Met 1264 Val presents milder OI signs (95,99). Met1264Val substitution located at the proα1(I) of C-propeptide is supposed to impede C-propeptide folding and chain association (99). Also, OI patients in C-terminal of type I collagen genes have various phenotypes from mild to lethal forms.

One special patient with OI/EDS was identified owing to c.4006-1G > A mutation in COL1A1 and biallelic missense variants in TNXB (p.Val1213Le, p.Gly2592Ser), he presented with severe muscular hypotonia, multiple fractures, and joint hyperflexibility (100). Homozygous mutation of splice site IVS 46 +2T>C caused non-functional COL1A2 alleles, patient with this mutation showed clinical features of generalized joint hypermobility, foot deformities, muscle hypotonia, blue sclerae and fracture history (73).

3.2.4. Caffey disease

Caffey disease, also known as infantile cortical hyperostosis (ICH), is caused by COL1A1 gene mutation of c.3040C>T and leads to an arginine to cysteine substitution at position 836. It rarely appears after 5 months of age and generally is restored spontaneously by 2 years old. Cortical thickening (hyperostosis) and subperiosteal new bone formation are the main findings of radiography. Tender swelling of long bone and inflammatory reactions often accompany the illness (101). Short stature and persistent bony deformities in 5 individuals from a three-generation Thai family are reported, which expands phenotypes of Caffey disease (102). Lethal or severe prenatal Caffey disease, complied with polyhydramnios, long bone deformity, and rib fractures, which could be misdiagnosed as lethal osteogenesis imperfecta (103-105). Two Caffey disease patients were reported to have overlapping phenotypes of joint hyperlaxity, hyperextensile skin and inguinal hernias resembling type III EDS (13).

Molecular mechanisms for Caffey disease are still unclear. Mutation of c.3040C>T locates in the sequence of interleukin-2 binding site and the amyloid protein precursor (APP), the gap region of major ligand binding regions (106-108). Arg836Cys substitution leads to increased disulfide crosslinking within or between collagen fibrils. Hence, widened and less densely packed collagen fibrils was observed from cultured skin fibroblast from Caffey disease (13). Meanwhile, thermal stability of collagen is reduced when an arginine residue was replaced in the X position of classic Gly-X-Y repeating pattern of triple helix collagen (109). It is also proposed that this typical mutation may regulate COX2/PGE axis mediated by unfolded protein response (110).

4. Conclusion

In conclusion, genetic and phenotypic overlap of OI, aEDS, cvEDS and Caffey diseases caused by type I collagen genes are outlined. Position preference mutations in COL1A1 and COL1A2 genes are highly related with typical phenotypes and diseases with different molecular mechanisms. Mutations of aEDS are limited to intron 5 and 6 of type I collagen genes. OI/EDS mutations locate predominately in the C-terminal and N-terminal region of exon 7 to exon 14. Considering the overlap mutation distribution of OI and EDS, it is a challenge to define disease with atypical phenotypes and atypical mutation distribution in type I collagen genes. Hence, clinical symptoms in detail for these diseases are extremely valuable. Meanwhile, to further analyze the heterozygosity in diseases like Caffey disease, which have a p.Gly188Asp mutation, as well as other diseases presenting clinical manifestations with the same pathogenic gene mutations, penetrance, expressivity and epigenetic regulation inheritance should be considered and combined with phenotype/genotypes of rare genetic syndromes. In all, genotypic and phenotypic relationships and molecular mechanisms for type I collagen-related inherited diseases will be further enriched and revealed as the number of rare diseases cases increases. Novel methods for classification will be useful for identification of rare diseases with atypical symptoms.

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Laparoscopic treatment of median arcuate ligament syndrome

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Summary

Median arcuate ligament syndrome (MALS) refers to a clinical syndrome caused by compression of the median arcuate ligament due to the fibers of this ligament that connect the diaphragmatic crura on the two sides of the aortic foramina, forming the anterior edge of the aortic foramina. If MALS is suspected, invasive digital subtraction angiography and computed tomography angiography or magnetic resonance angiography (MRA) can be used to verify the location of the celiac trunk. A disrupted or increased blood flow in the proximal end of the celiac trunk can be detected with doppler ultrasound, indicating stenosis. Treatment needs to alleviate celiac trunk compression. A common procedure involves separation of the ligament fibers and other surrounding tissues around the beginning of the celiac trunk. This can be achieved by either laparotomy or laparoscopic surgery. Patient prognosis is good, with a cure rate of about 80%.

Keywords: Median arcuate ligament syndrome, laparoscopic treatment

1. Introduction

The median arcuate ligament is the fibrous ligament that connects the diaphragmatic crura on the two sides of the aortic foramina, forming the anterior edge of the aortic foramina. The celiac trunk mostly starts below the median arcuate ligament and then divides into the hepatic artery, splenic artery, and left gastric artery. In 10% to 24% of the population, the celiac trunk is adjacent to the median arcuate ligament and may be compressed by the ligament, resulting in reduced blood supply for the corresponding organ and symptoms (1). Katz-Summercorn et al. (2) found that in 92.6% of 99 autopsies, the celiac trunk is adjacent to the median arcuate ligament and may be compressed by the ligament, resulting in reduced blood supply for the corresponding organ and symptoms (1). Katz-Summercorn et al. (2) found that in 92.6% of 99 autopsies, the celiac trunk is adjacent to the median arcuate ligament (the distance could not be measured), and in 33.7% of those cases the celiac trunk was compressed or distorted.

Median arcuate ligament syndrome (MALS), also known as celiac artery compression syndrome or Dunbar syndrome, mainly refers to a clinical syndrome caused by compression of the median arcuate ligament. MALS was first postulated in the 1960s (3,4) and was commonly seen in slender women ages 20 to 40 years (1). The typical triad of MALS syndromes is postprandial abdominal pain, weight loss, and an abdominal vascular murmur, but the appearance of these 3 symptoms at the same time is not common; other manifestations include anorexia, nausea, vomiting, diarrhea, and fatigue. Symptoms may be aggravated after exercise or when the body is in a certain position. The clinical manifestations after surgery are abdominal pain (80%), weight loss (48%), an abdominal vascular murmur (35%), nausea (9.7%), and diarrhea (7.5%) (5). The complications of MALS include gastroparesis and pancreatic duodenal aneurysm, etc., the mechanism of which includes long-term chronic ischemia, development of collaterals, and a long-term extensive decrease in blood flow (6-8). The diagnosis of MALS requires clinical manifestations and imaging studies and ruling out other causes that may lead to similar symptoms. Treatment is primarily performed by releasing the median arcuate ligament, removing the abdominal nerve plexus, and/or selective vascular reconstruction.
2. Etiology and pathogenesis of MALS

The pathogenesis of MALS has not been definitively determined and may include the mechanisms described below.

The starting point of the celiac trunk is too high or the median arcuate ligament is too long, compressing the celiac trunk, or abdominal ganglia are fused (including the superior mesenteric ganglia), compressing the celiac trunk (9). The compressed celiac trunk may cause limited blood flow and organ ischemia. Evidence to support the hypothesis of celiac trunk compression is that stenosis of the proximal end of the celiac trunk is found in the form of a hook in some patients during imaging studies; other findings are post-stenotic hemangiectasis and development of collaterals. The symptoms may resolve after release of the ligament during surgery. A study was conducted to determine whether the gastric mucosa in patients with MALS was ischemic by ascertaining gastric tension (10). When surgery was performed to release the ligament or intra-abdominal vascular reconstruction was also performed, ischemia was significantly alleviated.

In patients with conventional chronic mesenteric ischemia, however, the obstruction or severe stenosis of at least two of the three mesenteric vessels may result in symptoms of abdominal pain due to the presence of extensive collaterals carrying intestinal blood; however, the superior mesenteric artery and inferior mesenteric artery may not be affected in patients with MALS since they can theoretically provide a sufficient blood supply to the intestines. Evidence contradicting the theory of ischemia also includes angiography of asymptomatic patients which indicated compression of the median arcuate ligament (11,12). When surgery is performed to relieve compression, not all symptoms are alleviated (5). Therefore, abdominal pain may be associated with compression and intermittent ischemia of the visceral plexus (13). The abdominal nerve plexus is adjacent to the median arcuate ligament, and its source consists of preganglionic visceral nerves, phrenic nerves as well as somatic nerve of the vagus nerve, the preganglionic nerves of parasympathetic nerves, and postganglionic nerves of the vagus nerve. Abdominal pain can be caused by vasoconstriction or direct stimulation of the sympathetic nerves when the celiac plexus is involved.

Some studies contend that delayed gastric emptying is involved in the occurrence of MALS (13,14). A study of gastric myoelectric activity in a patient may facilitate readjustment of the gastric electrical rhythm and relief of symptoms, improving emptying of the contrast agent after compression of the celiac trunk is relieved.

In addition, one report described MALS in a couple of identical twin sisters (15) while another described compression of the celiac trunk in a father and a daughter as well as three brothers (16), suggesting that genetic factors may be involved in the development of this syndrome.

3. Diagnosis and treatment of MALS

The diagnosis of MALS requires the combination of clinical manifestations and imaging studies and ruling out other etiologies such as gallbladder disease, peptic ulcer, appendicitis, and inflammatory bowel disease (17), but there are no uniform diagnostic criteria.

A commonly used form of screening for MALS is Doppler ultrasound (18). If blood flow is disrupted or increased in the proximal end of the celiac trunk, this indicates stenosis. Gruber et al. (19) used a peak systolic expiratory velocity greater than 350 cm/s and bending of the celiac trunk greater than 50° as criteria to identify MALS with a sensitivity of 83% and a specificity of 100%. Selecting a certain systolic expiratory velocity could improve the sensitivity of diagnosis because celiac trunk compression is more severe due to lateral migration of the aortic and celiac trunk (20,21) and because the diameter of the celiac trunk increased significantly at end-systole compared to that at end-diastole (21). The advantages of Doppler ultrasound include its lack of invasiveness and the ease with which it is performed. Although it cannot determine the status of vessels during inspiratory and expiratory phases, it can be performed in a sitting or standing position.

If MALS is suspected, invasive digital subtraction angiography (DSA) or non-invasive computed tomography angiography (CTA) and magnetic resonance angiography (MRA) can be used to verify the location of the celiac trunk.

DSA can detect the presence of proximal stenosis and post-stenosis hemangiectasis and dilated vessel morphology and dynamic blood flow in the celiac trunk. In patients with MALS, the pancreaticoduodenal arterial arch in the superior mesenteric artery and retrograde filling of gastroduodenal artery into the celiac trunk can be observed. DSA can also detect the arterial pressure gradient across the origin of the celiac trunk, which helps to determine whether the celiac trunk is compressed (1). Kalapatapu et al. (22) proposed a new diagnostic method involving the administration of vasodilators via a catheter into the superior mesenteric artery during angiography. The method yields a positive result if the patient's symptoms reappear and the collateral blood reperfusion is absent. The study population consisted of 8 patients, 4 of whom had positive results. Three of those patients were cured after surgical alleviation of compression. Symptoms had other causes in 2 of the 4 patients who had negative results. Given the small sample size, the reliability of this method must be confirmed with additional observations.

CTA and MRA are forms of non-invasive imaging to identify celiac trunk compression. The advantages of CTA are that the examination is quick and inexpensive and that it better depicts intra-abdominal structures.
order to relieve compression. In some hospitals, removal of the celiac plexus is usually performed given ischemia leading to celiac trunk compression and involvement of the nerve plexus. In addition, celiac trunk dilation might be performed in a small number of procedures, or through vascular anastomosis, bypass, and reconstruction of the celiac trunk, which can be achieved by either laparotomy or laparoscopic surgery (5). Compared to laparotomy, laparoscopic surgery may reduce surgical trauma and patient hospitalization, improve the safety of the surgery, and ultrasound could be used to assist in confirming the opening of the celiac trunk (26,27). In addition, laparoscopic surgery could be performed with the da Vinci Surgical System as a form of robotic-assisted surgery (28,29), with improved surgical sensitivity and a wider visual field in comparison to conventional laparoscopic procedures.

Jimenez et al. (5) reviewed the English literature on MALS surgery and laparoscopic surgery between 1963 and 2012 and they analyzed postoperative outcomes in 400 patients, procedure details, and intraoperative and postoperative complications. The procedure was mainly the release of the median arcuate ligament; the celiac ganglia were removed or blood flow in the celiac trunk was restored in some patients. Results indicated that 85% (339/400) of patients had immediate postoperative relief of symptoms, and the rate of symptom recurrence was 6.8% (19/279) in patients who underwent a laparotomy and 5.7% (7/121) in those who underwent laparoscopic surgery. Restoring blood flow was not more likely to relieve symptoms compared to simple release of the median arcuate ligament; the incidence of complications was 11.6% for laparoscopy.

With the development of CT equipment and CT image processing technology, thin-layer multi-slice spiral CT combined with a 3D reconstruction technique can clearly depict the aorta and its branches, and it has basically replaced traditional aortography (23). The sagittal view is the ideal orientation in which to observe the proximal end of the celiac trunk, and celiac trunk compression can be observed in the anterior side at the proximal end in patients with MALS. Hook-like stenosis (which can be distinguished from stenosis due to other causes, such as atherosclerotic stenosis) is evident in the celiac trunk. The morphology of the median arcuate ligament and collateral vessels can be observed in addition to three-dimensional rendering (1). CTA is often performed in the inspiratory phase because celiac trunk compression is more severe in the expiratory phase; evidence of compression in the inspiratory phase suggests MALS (Figure 1). Manghat et al. used ECG-gated CTA to ascertain changes in celiac trunk flow dynamics and abdominal organ supply in the cardiac cycle, but its diagnostic significance in MALS should be studied further (21). Compared to CTA, MRA takes longer for a scan and is more expensive, but its spatial resolution and depiction of calcified plaques are inferior to CTA. However, the absence of radioactivity during MRA and the fact that the contrast agent does not contain iodine make it suitable for use in children, pregnant women, patients allergic to iodine-containing contrast media, and patients with impaired renal function (24).

4. Treatment of and prognosis for MALS

Due to the different definitions of MALS and different criteria for subject selection, the appropriate treatment for MALS is a subject of debate.

In light of the pathogenicity of MALS, treatment needs to alleviate celiac trunk compression. Since compression of the celiac trunk is physical rather than due to intravascular lesions, such as atherosclerosis, use of percutaneous balloon dilation or placement of a stent is not feasible (25). A common procedure is separation of the ligament fibers and other surrounding tissues around the beginning of the celiac trunk in order to relieve compression. In some hospitals, removal of the celiac plexus is usually performed given ischemia leading to celiac trunk compression and involvement of the nerve plexus. In addition, celiac trunk dilation might be performed in a small number of procedures, or through vascular anastomosis, bypass, and reconstruction of the celiac trunk, which can be achieved by either laparotomy or laparoscopic surgery (5). Compared to laparotomy, laparoscopic surgery may reduce surgical trauma and patient hospitalization, improve the safety of the surgery, and ultrasound could be used to assist in confirming the opening of the celiac trunk (26,27). In addition, laparoscopic surgery could be performed with the da Vinci Surgical System as a form of robotic-assisted surgery (28,29), with improved surgical sensitivity and a wider visual field in comparison to conventional laparoscopic procedures.

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and 6.5% for open surgery. The common complications of laparoscopy mainly included bleeding and pneumothorax, postoperative complications included pancreatitis and gastroparesis (in 1 patient each), and laparoscopy had to be converted to open surgery in 9.1% of patient (11/121) due to bleeding. The primary complications of open surgery included post-operative vascular thrombosis, stroke, and gastroesophageal reflux. There were no procedure-related deaths due to either procedure. Laparoscopic surgery is performed at the authors’ facility to treat MALS, and patient prognosis is good (Figure 2).

Reilly et al. (30) analyzed factors that may affect the outcomes of surgery for celiac trunk compression, and results suggested that factors for a better prognosis included postprandial abdominal pain (81% cure rate), an age between 40 and 60 years (77% cure rate), weight loss greater than 20 pounds (67% cure rate), and no history of mental illness or alcohol abuse. However, this study can only be used as reference due to the small sample, the fact that multivariate statistical analysis was not performed, different procedures performed, and different definitions of "cured." The key to a good prognosis is the identification of "true" patients and the development of appropriate treatments. Given neurogenic involvement in MALS, Sultan et al. (31) contended that the abdominal nerve plexus could be routinely removed in addition to release of the ligament. Additional clinical experience is needed to devise an "Optimal Treatment." If an aneurysm has formed in a collateral, it might rupture, so the pathogenesis of MALS needs to be quickly determine and treatment needs to be performed as soon as possible.

References


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A preliminary study on the mechanism of skeletal abnormalities in Turner syndrome using inducing pluripotent stem cells (iPS)-based disease models

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Summary

Osteoporosis represent one of main characteristics of Turner syndrome (TS), a rare diseases caused by aberrant deletion of X chromosomes, however, the underlying pathological mechanism remains unknown yet. In this study, we used pluripotent stem cells (iPSCs) derived from a Turner syndrome patient and a health control to induce functional osteoblasts and osteoclasts, in order to compare their difference in these two differentiation. We successfully produced functional osteoblasts and osteoclasts from iPSCs through embryoid bodies (EBs) and mesoderm stages, as demonstrated obvious mineralized nodules and multi-nuclear giant cells with positive tartrate-resistant acid phosphatase (TRAP) staining, and significant up-regulated differentiation marker genes. Interestingly, we found that there was no significant difference in phenotype and marker genes expression between osteoblasts from Turner syndrome and healthy control iPSCs. In contrast, Turner syndrome showed increased osteoclastogenesis compared to the healthy control indicating higher frequency of multi-nuclear TRAP staining cells and elevated osteoclast marker genes TRAP, MMP9, CA2, OSCAR. Therefore, our results suggest that the low bone density of Turner syndrome patients may be caused by aberrant osteoclast differentiation, and further investigation towards osteoclast function under Turner syndrome is deserved.

Keywords: Turner syndrome, induced pluripotent stem cells, osteoblasts, osteoclasts

1. Introduction

Turner syndrome is a relatively common type of human chromosome aberration with an incidence rate of 1/2,500 (1). It is also called congenital ovarian dysplasia syndrome, which is caused by total or partial deletion of X chromosomes in all or part of the cells. Monosomy 45, X accounts for about 45% of TS, while the remaining patients exhibit multiple chimeras and structural abnormalities (2).

Primary ovarian insufficiency and short stature are prominent clinical features of TS, osteoporosis and fractures are important consequences (3). The cause of skeletal fragility of TS may be multifactorial, for example chromosomal abnormalities, acquired osteoporosis and visual spatial cognitive dysfunction, and increased risk of fractures due to impaired balance (4). Several previous studies have found a significant reduction in bone density in TS using dual-energy X-ray absorptiometry (DXA) (5). Although recombinant growth hormone treatment has been given to TS patients to treat short stature in childhood, the effectiveness of this treatment for bone mineral density improvement remains controversial.

The underlying cellular and molecular mechanisms of osteoporosis, in particular, the role of two most important players, the functions of osteoblasts and osteoclasts are still unknown, as well as important. However, the unavailability of tissues from patients is
a main bottleneck in this field. In this study, we have used induced pluripotent stem cells to establish an osteoblast model and an osteoclast model to mimic the pathological process of bone remodeling in vitro to investigate the phenotype variation of these cells, and to explore their biological significance.

2. Materials and Methods

2.1. TS and normal iPSCs lines

One human iPSCs line derived from fibroblasts of a TS patient was obtained from Sidansai Biotechnology Company (Shanghai, China), an 8-year-old female with a 45,X karyotype. Cells derived from an iPSCs line from a healthy volunteer were established from a 25-year-old healthy woman. Undifferentiated iPSCs were seeded on six-well plates (Sorfa, Zhejiang, China) coated with Matrigel (Corning, Bedford, MA, USA) and cultured using mTesR medium (StemCell Technologies, Vancouver, BC, Canada). These two iPSCs were established with informed consent. The study procedures of this study were approved by the ethnic committee of Shandong Medical Biotechnological Center.

2.2. Induction of osteoblasts from TS and normal iPSCs

In order to induce differentiation, the two iPSCs lines were first cultured in six-well plates for 5-7 days, when the cells were overgrown, the embryoid bodies (EBs) were obtained by a mechanical method, and cultured in a low attachment six-well plate (Corning, Kennebunk, ME, USA) with added EBs medium (Osinglay, Guangzhou, China) (day 0). After 2 days culture, EB medium was changed with fresh EB medium, all-trans retinoic acid (RA) (MCE, Monmouth Junction, NJ, USA) was added after 48h to induce differentiation of EBs to mesoderm. At day 8, we digested EBs with BioC-PDE1 Digestive Enzyme (Osinglay, Guangzhou, China) allowing cells to grow adherently. The cells were further cultured 1 day, and the osteogenic medium was replaced (Cyagen, Guangzhou, China). The iPSCs-derived osteoblasts were induced for 7 days, 14 days, and 21 days, respectively.

2.3. Induction of osteoclasts from TS and normal iPSCs

The procedures for iPSCs and EBs culture were the same for induction of osteoclasts. For osteoclast differentiation, EBs were generated using 6-well low cluster plates in STEMDIFF APEL 2 medium (StemCell Technologies, Vancouver, BC, Canada) supplemented with 50 ug/mL ascorbic acid (Sigma, Louis, MO, USA), 4 × 10^{-3} M thioglycerol (Sigma, Louis, MO, USA), 2 mM glutamine (Sigma, Louis, MO, USA), 10 ng/mL human basic fibroblast growth factor (bFGF) (R&D Systems Inc., Minneapolis, MN, USA), then incubating for 24 hours in an environment of 37°C, 5% O_2, 5% CO_2, 90% N_2, 5 ng/mL human basic fibroblast growth factor (bFGF) (R&D Systems Inc., Minneapolis, MN, USA) was added for 3 days to induce mesoderm formation. Cells were cultured in STEMDIFF APEL 2 medium with 10 ng/mL human vascular endothelial growth factor (VEGF) (R&D Systems Inc., Minneapolis, MN, USA), 1 ng/mL bFGF, 10 ng/mL human interleukin-6(IL-6) (R&D Systems Inc., Minneapolis, MN, USA), 40 ng/mL human interleukin-3 (IL-3) (R&D Systems Inc., Minneapolis, MN, USA), 5 ng/mL human interleukin-11 (IL-11) (R&D Systems Inc., Minneapolis, MN, USA), 100 ng/mL human stem cell factor (HSCF) (R&D Systems Inc., Minneapolis, MN, USA) to promote hematopoietic cell growth. After 5 days culture, cells were moved to a 5% CO_2 environment and 10 ng/mL VEGF, 10 ng/mL IL-6, 40 ng/mL IL-3, 5 ng/mL IL-11, 100 ng/mL HSCF, 4 U/mL human erythropoietin (EPO) (R&D Systems Inc., Minneapolis, MN, USA), 50 ng/mL human thrombopoietin (TPO) (R&D Systems Inc., Minneapolis, MN, USA), 10% FCS (Gibco, Carlsbad, CA, USA) were added to STEMDIFF APEL 2 medium to culture for 10-14 days to promote hematopoietic cell maturation and myeloid cell expansion. Then we digested the cells and cultured on six-well plates in IMDM (Gibco, Carlsbad, CA, USA) containing 30 ng/mL M-CSF (R&D Systems Inc., Minneapolis, MN, USA), and 50 ng/mL RANKL (R&D Systems Inc., Minneapolis, MN, USA) and 10% FCS for 10 to 14 days.

2.4. Real-time quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR was used to semi-quantitatively analyze the marker genes for osteoblasts (ALP, RUNX2, COL1A1, OCN), hematopoietic stem cells (CD34, CD45, RUNX1, GATA2), mononuclear macrophages (TNFALP3, THBS1, RUNX1) and osteoclasts (MMP9, OSCAR, CATK, CTR). The primers are listed in Table 1. Briefly, total RNA was extracted using Trizol reagent (Gibco, Carlsbad, CA, USA) and then we used the reverse transcription kit (Toyobo, Osaka, Japan) to synthesize cDNA. RT-qPCR reactions were performed according to the ratio of Sybr Green Realtime PCR Master Mix (Toyobo, Osaka, Japan) : RNAase-free water (Tiangen, Beijing, China): forward: reverse: cDNA at 5:2:1:1:1. Each reaction was run on Light Cycler® 480 (Roche Applied Science, Mannheim, Germany).

2.5. Alizarin red staining

Alizarin red staining was performed using STAINING Kit (GenMed Sciences Inc., Arlington, MA, USA). After 50 days of induction from iPSCs, differentiated
osteoblasts were stained for mineralized nodules. Briefly, the cells in one well of a 6-well plate was washed twice with solution A, and then cell fixative B reagent was used to fix the cells for 10 minutes. After that, the well was washed twice using cell clearing solution A. Then, staining solution C was added to the culture plate and incubated for 20 minutes at room temperature.

2.6. TRAP staining

The osteoclast activity was evaluated by TRAP staining using a commercial kit (Sigma, Louis, MO, USA). The cells were washed twice with PBS (Gibco, Carlsbad, CA, USA), then fixed with paraformaldehyde (Solarbio, Beijing, China), and the chromogenic reagent was prepared according to the manufacturer’s instructions and added to the well for 1 hour. Staining results were observed under the microscope.

2.7. Statistics

To determine the differences between groups, student’s t-test was carried out. We used SPSS statistics 19 to calculate data, with significance accepted at \( p < 0.05 \). Mapping with Graphpad Prism 6.0.

3. Results

3.1. Comparison of osteoblast differentiation of iPSCs from TS and normal control

The procedure of osteoblast induction from iPSCs is shown in Figure 1A. Through EBs stage, typical osteoblasts with mineralizing ability could be established (Figure 1B). Marker genes at 7 days, 14 days and 21 days of osteogenesis induction, and mineralized nodule formation at 50 days from TS-iPSCs and normal-iPSCs were compared. As seen in Figure 2A, in all 3 stages, expression of osteogenesis marker genes seems to be higher in the TS-derived osteoblasts than the normal control. But there is no obvious difference in mineralization formation in

### Table 1. List of primers RT-qPCR

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequence</th>
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<tbody>
<tr>
<td>ALP</td>
<td>Forward: CCGTGCACTCTATCTTGG&lt;br&gt;Reverse: GCCATACAGGATGCGATTA</td>
</tr>
<tr>
<td>RUNX2</td>
<td>Forward: AGCAAGGTTTCAAGATCTGTAGAT&lt;br&gt;Reverse: TTGTGAAAGCAGGTTATGGTCAA</td>
</tr>
<tr>
<td>COL1A1</td>
<td>Forward: CCGTGAAAGAATGGAGATGA&lt;br&gt;Reverse: ACTGAAACTCTCTGTGCTCCCTCA</td>
</tr>
<tr>
<td>OCN</td>
<td>Forward: AACGAGCCAGCGGCTACCT&lt;br&gt;Reverse: AACCTGTCACAGTCCGAGGAT</td>
</tr>
<tr>
<td>CD34</td>
<td>Forward: TGACCGGCTTGGGTC&lt;br&gt;Reverse: CCCTTGGTACTGAACTCTGGG</td>
</tr>
<tr>
<td>CD45</td>
<td>Forward: CGAAGAGCCGTTCCAGAGGGAC&lt;br&gt;Reverse: AAATGACAGCGCTTCCAGAAGGGC</td>
</tr>
<tr>
<td>RUNX1</td>
<td>Forward: CCGAGAACCCTGAAGACATC&lt;br&gt;Reverse: GCCTGACCCTATGGCTG</td>
</tr>
<tr>
<td>GATA2</td>
<td>Forward: GGCTAGGGAACAGATCGAGC&lt;br&gt;Reverse: GCAGCAGGGCCAGCAGG</td>
</tr>
<tr>
<td>TNALP3</td>
<td>Forward: TCAAATCTTGTCGAAAGGTC&lt;br&gt;Reverse: CCAAAGTCTGTGTGCTGAAAC</td>
</tr>
<tr>
<td>THBS1</td>
<td>Forward: AGACGCTCTCAACAGAGAGC&lt;br&gt;Reverse: TGTCAGTGGTTCAAAAGACAA</td>
</tr>
<tr>
<td>RUNX1</td>
<td>Forward: CCGAGAACCCTGAAGACATC&lt;br&gt;Reverse: GTCTGACCCTATGGCTG</td>
</tr>
<tr>
<td>Mmp9</td>
<td>Forward: GACACATCCACTCAGCCAGG&lt;br&gt;Reverse: GCCAACCCAGGTGTAACACATA</td>
</tr>
<tr>
<td>OSCAR</td>
<td>Forward: CCGCTTCACTACCACCCTA&lt;br&gt;Reverse: GAAGAGAAGGGAGGAGGATCT</td>
</tr>
<tr>
<td>CATK</td>
<td>Forward: CAGTGAAGAAGGTTGTCAGGA&lt;br&gt;Reverse: AGACTCTGTCGGGCTCACCTT</td>
</tr>
<tr>
<td>CTR</td>
<td>Forward: TTTCAGGATGAAACAGTCCTGACATA&lt;br&gt;Reverse: AATGCTATGACCGAATGCAAAGCAT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: GCACCGTCAAGGCCCAGCCAG&lt;br&gt;Reverse: ATGGTGAGGAGGGAGCCAGT</td>
</tr>
</tbody>
</table>

To determine the differences between groups, student's \( t \)-test was carried out. We used SPSS statistics 19 to calculate data, with significance accepted at \( p < 0.05 \). Mapping with Graphpad Prism 6.0.
osteoblasts at 50 days (Figure 2B).

3.2. **Comparison of osteoclast differentiation of iPSCs from TS and normal control**

Figure 3A demonstrated our stepwise procedure to induce TS- and normal- iPSCs to osteoclasts through hematopoietic cell and mononuclear macrophage stages. The induction at all stages was successful. As shown in Figure 3B and Figure 3C, after induction, hematopoietic cell-related genes CD34, CD45, RUNX1, GATA2 and mononuclear macrophage-associated genes PU.1, TNFALP3, THBS1 were significantly elevated. At the end of induction, significantly elevated osteoclast marker genes TRAP, CA2, MMP9, OSCAR were validated (Figure 3D).

When the extent of osteoclast differentiation of TS- and normal- iPSCs was compared, the TS-iPSCs derived osteoclasts indicated a stronger TRAP staining intensity and density than the normal iPSCs-derived control (Figures 4A and 4B). Consistent with the TRAP staining results, the expression marker genes of osteoclast differentiation were also significantly increased in the TS-iPSCs derived osteoclasts than that in the normal iPSCs-derived control (Figure 4C). These findings support that TS osteoclasts may have a higher osteolytic activity than normal osteoclasts.

4. **Discussion**

Abnormal skeletal differentiation is one of main characteristics of TS, which has been always neglected. It has been proposed that loss of function of some genes, in particular SHOX, may contribute to the pathology of TS. Ibarra-Ramírez et al. confirmed that SHOX and VAMP7 showed the most obvious gene dose changes in the pseudo-autosomal region of TS (6). Regarding the short stature, it has been suggested that the homeobox gene SHOX in the pseudoautosomal region is a major player, and the haplotype deficiency of this gene leads to growth disorders in TS (7,8). In addition, Kosho et al. have reported that SHOX haploinsufficiency can lead to additional TS skeletal abnormalities such as short fourth metacarpars and cubitus valgus (9). Clement-Jones et al. showed that SHOX nonsense mutations can lead to rich internal phenotypic variability in some of these skeletal features (10).

Osteoporosis is another clinical manifestation, which makes TS patients more prone to fractures than the general population, but the etiology of decreased bone density has not been elucidated. The bone formation process can be influenced by several factors, such as genetic inheritance, gender, ethnicity and endocrine activity (endogenous factors), as well as nutrition and physical activity (exogenous factors) (11). About 60% of
the risk of osteoporosis may be due to damage to mineral bone at the beginning of adulthood (12). In healthy adults, bone remodeling is a highly coordinated process involving bone resorption and bone formation and is regulated by both osteoblasts and osteoclasts. Bone development and remodeling involves and depends on the interaction between bone cell precursors, bone cells, extracellular matrix molecules, growth factors, immune system and humoral factors (13).

For the scarcity of cases surgical procedures are rarely performed for TS patients, therefore, direct obtainment of bone tissues from patients is difficult. The application of iPSCs can provide a good model for rare diseases for they can be induced to form different disease-related cell types. In order to analyze the cause of abnormal bone density in TS patients, in this study,
using fibroblasts and urine cell derived iPSCs from TS and healthy cases, we established two bone remodeling cell types, osteoblasts and osteoclasts, which were validated by functional analyses and differentiation marker detection.

For the first time, our results found that there is an obvious difference in osteoclast but not osteoblast differentiation between TS patients and healthy controls. Osteoclasts from TS iPSCs demonstrated a higher activity than normal iPSCs derived osteoclasts, which can result in unbalance in bone metabolism.

Lauren J. Massingham et al. found that IGFBP5 overexpression in the Turner syndrome transcriptome (14), and its overexpression increases the formation of osteoclasts (15). Bisphosphonates are effective in preventing fragility fractures, and we can consider using it for the treatment of Turner syndrome (16). Together with the above findings, our data also supported that these patients also benefit from other osteoclast-targeted strategies.

In conclusion, this study revealed that osteoclasts but not osteoblasts from TS-derived iPSCs are abnormal which is helpful to explain the low bone density of TS patients. In future research, efforts to explore the role of genes and pathways related to aberrant osteoclast differentiation in TS will be investigated.

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Microglia express GPNMB in the brains of Alzheimer's disease and Nasu-Hakola disease

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Summary

Glycoprotein non-metastatic melanoma protein B (GPNMB) is a type I transmembrane glycoprotein first identified in low-metastatic human melanoma cell lines as a regulator of tumor growth. GPNMB is widely expressed in various tissues, where it is involved in cell differentiation, migration, inflammation/anti-inflammation, tissue regeneration, and neuroprotection. GPNMB is identified in microglia of adult rat brains, neurons and astrocytes of GPNMB transgenic (Tg) mouse brains, and motor neurons of amyotrophic lateral sclerosis (ALS) patients. Nasu-Hakola disease (NHD) is a rare autosomal recessive disorder, characterized by progressive presenile dementia and formation of multifocal bone cysts, caused by genetic mutations of either TYROBP (DAP12) or TREM2. TREM2 and DAP12 constitute a receptor/adaptor signaling complex expressed exclusively on osteoclasts, dendritic cells, macrophages, and microglia. Pathologically, the brains of NHD patients exhibit leukoencephalopathy, astrogliosis, accumulation of axonal spheroids, and remarkable activation of microglia predominantly in the white matter of frontal and temporal lobes and the basal ganglia. At present, molecular mechanisms responsible for development of leukoencephalopathy in NHD brains remain totally unknown. Recent evidence indicates that disease-associated microglia (DAM) that cluster around amyloid plaques express high levels of GPNMB in Alzheimer's disease (AD) brains. Because microglia act as a key regulator of leukoencephalopathy in NHD brains, it is proposed that GPNMB expressed on microglia might play a protective role in progression of leukoencephalopathy possibly via active phagocytosis of myelin debris. In the present study using immunohistochemistry, we have attempted to clarify the expression of GPNMB in NHD brains, compared with AD brains. We found that microglia accumulating in the white matter express an intense GPNMB immunoreactivity in both NHD and AD brains, suggesting that the accumulation of GPNMB-immunoreactive microglia is a general phenomenon in neurodegenerative brains.

Keywords: Alzheimer's disease, GPNMB, leukoencephalopathy, microglia, Nasu-Hakola disease, osteoactivin

1. Introduction

Glycoprotein non-metastatic melanoma protein B (GPNMB) is a type I transmembrane glycoprotein first identified in low-metastatic human melanoma cell lines as a regulator of tumor growth, alternatively named osteoactivin (1). GPNMB is composed of an N-terminal signal peptide, a RGD motif, a polycystic kidney disease (PKD) domain, and a proline-rich repeat domain (PRRD) in its extracellular domain (ECD), a single-pass transmembrane domain, and a short cytoplasmic tail that possesses a half immunoreceptor tyrosine-based activation motif (hemITAM) and a dileucine motif (2,3). The transmembrane segment of GPNMB is proteolytically cleaved at a dibasic motif in the juxamembrane region by a disintegrin and
metalloproteinatease (ADAM) family of proteases and matrix metalloproteinasases (MMPs) in a process called ectodomain shedding, which results in the release of a soluble form of the GPNMB extracellular domain (4,5). The RGD motif serves as an integrin-binding motif, while the PKD domain mediates protein-protein and protein-carbohydrate interactions. The hemITAM motif induces signal transduction following ligand binding. The dileucine motif mediates rapid internalization of GPNMB from the plasma membrane processed for lysosomal/endoosomal targeting. GPNMB is a heavily glycosylated protein, possessing 12 putative N-glycosylation sites within its extracellular domain (2,3). GPNMB is detected by immunoblot as two glycosylated high molecular weight isoforms (97-kDa, 116-kDa) (2). GPNMB is located not only at the plasma membrane but also in the perinuclear cytoplasmic regions, the endoplasmic reticulum (ER), and the Golgi apparatus (2,6,7). Extracellular fragments of GPNMB induce the production of matrix metalloproteinase-3 (MMP-3) and activation of ERK1/2 and p38 in mouse fibroblasts (8).

GPNMB plays a role in regulation of the homeostasis in various tissues and cells, such as skeletal muscle, bone, the hematopoietic system, the nervous system, epithelial cells, osteoblasts, osteoclasts, macrophages, and dendritic cells (9,10). GPNMB is involved in cell differentiation, migration, inflammation/anti-inflammation, tissue regeneration, and neuroprotection (2,3). GPNMB acts on osteoblasts to stimulate differentiation, leading to bone mineral deposition (11). DBA/2J mice that have a GPNMB mutation resulting in a truncated nonfunctional protein show less bone formation (12).

On the other hand, GPNMB acts as a negative regulator of osteoclastogenesis (13). GPNMB is shown to inhibit osteoclast differentiation through binding to CD44 and inhibiting ERK activation. GPNMB negatively regulates the inflammatory responses of macrophages in a mouse model of experimental colitis (14). Using primary mouse astrocytes from CD44 knockout mice, it was indicated that the anti-inflammatory effects of GPNMB require binding to CD44 (15).

In the central nervous system (CNS), GPNMB is expressed in the cerebrum, cerebellum, brain stem, and spinal cord of adult rats (16). However, at present, the precise cell type expressing GPNMB in the human CNS remains unknown. Purified human oligodendrocytes by panning with anti-galactocerebroside (GalC) antibody express GPNMB (https://www.brainrnaseq.org). GPNMB expression is elevated in the substantia nigra of Parkinson’s disease (PD) patients compared with age-matched controls (17). GPNMB is greatly expressed in motor neurons with extracellular deposits in the spinal cord of amyotrophic lateral sclerosis (ALS) (18,19). A subpopulation of Iba1-positive microglia that cluster around amyloid plaques expresses high levels of GPNMB in Alzheimer’s disease brains (20).

Nasu-Hakola disease (NHD), also designated polycystic lipomembranous osteodysplasia with sclerosing leukencephalopathy (PLOSL), is a rare autosomal recessive disorder, characterized by progressive presenile dementia and formation of multifocal bone cysts, caused by loss-of-function mutations of either TYROBP (DAP12) or TREM2 (21). TREM2 and DAP12 constitute a receptor/adaptor signaling complex expressed exclusively on osteoclasts, dendritic cells, macrophages, and microglia. Although NHD patients are clustered in Japan and Finland, approximately 200 NHD cases are presently reported worldwide. Clinically, the patients with NHD show recurrent bone fractures during the third decade of life, and a frontal lobe syndrome during the fourth decade of life, and progressive dementia and death until the fifth decade of life (22). Pathologically, the brains of NHD patients exhibit extensive demyelination designated leukencephalopathy, astrogliosis, accumulation of axonal spheroids, and remarkable activation of microglia predominantly in the white matter of frontal and temporal lobes and the basal ganglia (23). At present, molecular mechanisms responsible for development of leukencephalopathy in NHD brains remain totally unknown. Because NHD is a pathological entity of microgliopathy where microglia act as a key regulator of leukencephalopathy, we propose the hypothesis that GPNMB expressed on microglia might play a protective role in progression of leukencephalopathy possibly via active phagocytosis of myelin debris in NHD brains. In the present study, we have attempted to clarify the expression of GPNMB in NHD brains compared with AD brains.

2. Materials and Methods

2.1. Human brain tissues

The brain autopsies were performed at the National Center Hospital, National Center of Neurology and Psychiatry (NCNP), Japan, Kohnodai Hospital, National Center for Global Health and Medicine (NCGM), Japan, and affiliated hospitals of Research Resource Network (RRN), Japan. The comprehensive examination by established neuropathologists (YS and TI) validated the pathological diagnosis. Written informed consent was obtained in all cases. The Ethics Committee of the NCNP for Human Brain Research, the Ethics Committee of the NCGM on the Research Use of Human Samples, and the Human Research Ethics Committee (HREC) of the Meiji Pharmaceutical University (MPU) approved the present study.

For immunohistochemical studies, serial sections of the frontal lobe and the hippocampus were prepared from four subjects who died of non-neurological causes (NC), composed of a 63-year-old man who died of prostate cancer and acute myocardial infarction (NC1), a 67-year-
old man who died of dissecting aortic aneurysm (NC2), a 57-year-old man who died of alcoholic liver cirrhosis (NC3), and a 61-year-old man who died of rheumatoid arthritis with interstitial pneumonia (NC4), ten AD patients, composed of a 68-year-old woman (AD1), a 70-year-old woman (AD2), a 68-year-old woman (AD3), a 56-year-old man (AD4), a 59-year-old man (AD5), an 81-year-old man (AD6), a 68-year-old woman (AD7), an 80-year-old man (AD8), a 72-year-old man (AD9), and a 77-year-old woman (AD11), and five NHD patients, composed of a 42-year-old man (NHD1), a 48-year-old woman (NHD2), a 44-year-old man (NHD3), a 32-year-old woman (NHD4), and a 38-year-old man (NHD5). The homozygous mutation of a single base deletion of 141G (c.141delG) in exon 3 of DAP12 was identified in NHD1, NHD2, and NHD5, while the genetic analysis was not performed in NHD3 or NHD4. All AD cases were satisfied with the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) criteria for diagnosis of definite AD (24). They were categorized into stage C of amyloid deposition and stage VI of neurofibrillary degeneration, following Braak's staging (25).

2.2. Immunohistochemistry

After deparaffinization, tissue sections were heated in 10 mM sodium citrate buffer, pH 6.0 by autoclave at 110°C for 15 min in a temperature-controlled pressure chamber (Biocare Medical, Pacheco, CA, USA). They were treated at room temperature (RT) for 15 min with 3% hydrogen peroxide-containing methanol to block endogenous peroxidase activity. They were then incubated with phosphate-buffered saline (PBS) containing 10% normal rabbit serum at RT for 15 min to block non-specific staining, followed by incubation in a moist chamber at 4°C overnight with goat polyclonal anti-GPNMB antibody (AF-2550, R&D Systems, Minneapolis, MN, USA). The specificity of anti-GPNMB antibody was validated by Western blot analysis of recombinant human GPNMB protein which was cloned with the pEF6/V5-His TOPO vector (Thermo Fisher Scientific, Waltham, MA, USA) expressed in HEK293 cells.

After washing with PBS, tissue sections were incubated at RT for 30 min with horseradish peroxidase (HRP)-conjugated anti-goat secondary antibody (Nichirei, Tokyo, Japan), mouse monoclonal antibody against amyloid-β peptide (12B2; Immunobiological Laboratories, Gunma, Japan), mouse monoclonal antibody against phospho-tau (Ser202, Thr205) (AT8; Thermo Fisher Scientific), mouse monoclonal antibody against apolipoprotein E (apoE) (ab1906; Abcam, Cambridge, UK), mouse monoclonal antibody against GFAP (GA5; Nichirei), or mouse monoclonal antibody against NeuN (ab104224; Abcam), followed by incubation with alkaline phosphatase-conjugated anti-rabbit or anti-mouse secondary antibody (Nichirei) and exposure to Warp Red chromogen (Biocare Medical).

2.3. Quantification of GPNMB immunoreactivity

To quantify immunolabeled areas, the images derived from three fields of the frontal cortex or the subcortical white matter per each section were captured at a 200× magnification on the Olympus BX51 universal microscope. They were then processed for quantification by using ImageJ software (National Institute of Health, Bethesda, MD, USA). The differences in the GPNMB-positive areas among AD, NHD and NC subjects were evaluated statistically by one-way analysis of variance (ANOVA) followed by post-hoc Tukey's test.

3. Results

First, we validated the specificity of anti-GPNMB antibody AF-2550 by Western blot of a V5-tagged recombinant GPNMB protein expressed in HEK293 cells (~100-kDa, 120-kDa) (Figure 1A). Then, by

![Image 1](https://www.irdrjournal.com)

**Figure 1. Validation of the specificity of anti-GPNMB antibody.** Western blot analysis of a V5-tagged recombinant GPNMB protein with (A) anti-GPNMB antibody AF-2550, (B) anti-V5 antibody, and (C) anti-G3PDH antibody, as a loading control. (lane 1) non-transfectant and (lane 2) transfectant.
immunohistochemistry, we found that GPNMB is intensely expressed predominantly in amoeboid and hypertrophic microglia located in the subcortical white matter of the frontal lobe and the hippocampus of both AD and NHD brains (Figure 2, panels b, c, d; Figure 3, panels a, b). In contrast, a much smaller area was labelled with GPNMB in NC brains (Figure 2, panel a). In the frontal white matter of NHD, the area of GPNMB-expressing cells showed an 18.9-fold increase compared with NC ($p = 0.0035$) (Figure 4, panel a). In

![Figure 2. Immunohistochemistry of frontal white matter with anti-GPNMB antibody. (a) frontal white matter, NC, (b) hippocampus, AD, (c) frontal white matter, AD and (d) frontal white matter, NHD. Scale bars indicate (a, c, d) 50 μm and (b) 100 μm.](image)

![Figure 3. Immunohistochemistry of NHD brains with anti-GPNMB antibody. (a-d) NHD brains. (a, b) frontal white matter, (c) frontal cortex and (d) hippocampus. Scale bars indicate (a-d) 50 μm.](image)
the frontal cortex of AD, the area of GPNMB-expressing cells exhibited a 2.4-fold increase compared with NHD ($p = 0.0177$) and a 5.7-fold increase compared with NC ($p = 0.0027$) (Figure 4, panel b). Thus, GPNMB-immunoreactive area is greatest in the frontal white matter of NHD and in the frontal cortex of AD. The great majority of GPNMB-expressing cells were labeled with Iba1 but neither with GFAP nor NeuN (Figure 5, panels a-f).

**Figure 4.** GPNMB-immunolabeled areas of frontal white matter and frontal cortex. The differences in the GPNMB-positive areas among AD, NHD, and NC subjects were evaluated statistically by one-way analysis of variance (ANOVA) followed by post-hoc Tukey’s test. (a) Frontal white matter and (b) frontal cortex.

**Figure 5.** Double immunolabeling with anti-GPNMB antibody and cell type-specific antibodies. (a-f) AD, (a-c, f) hippocampus and (d, e) frontal cortex. Double immunolabeling of GPNMB (brown) with (a) Iba1 (red), (b) GFAP (red), (c) NeuN (red), (d) amyloid-β (red), (e) ApoE (red), or (f) AT8-tau (red). Scale bars indicate (a-c, e, f) 20 μm and (d) 50 μm.
a-c). In AD brains, the clusters of GPNMB-expressing microglia accumulated on amyloid-β-positive and APOE-immunolabeled plaques (Figure 5, panels d, e). In AD brains, phosphorylated tau immunoreactivity was often in close contact with GPNMB aggregates (Figure 5, panel f). In AD brains, the clusters of GPNMB-expressing amoeboid and hypertrophic microglia forming plaques were identified frequently in the frontal cortex and the hippocampus (Figure 6, panel a-d). In contrast, only a few clusters of GPNMB-expressing microglia were found in NHD brains (Figure 3, panels a, c). In AD brains, perivascular macrophages and some degenerating neurons occasionally expressed GPNMB, while reactive astrocytes rarely showed GPNMB immunoreactivity. In NHD brains, perivascular macrophages and a few neurons also expressed GPNMB (Figure 3, panels b, d).

4. Discussion

GPNMB is a widely expressed multifunctional protein that regulates cell differentiation, migration, inflammation/anti-inflammation, tissue regeneration, and neuroprotection. Previous studies showed that GPNMB is expressed on astrocytes and neurons in GPNMB Tg mice (26). In addition, GPNMB is expressed in motor neurons, but not in astrocytes or microglia in ALS patients (19). Recent evidence indicates that microglia express high levels of GPNMB. GPNMB-positive cells express most frequently the microglia/macrophage marker OX42, and occasionally the radial glia marker RC2 or the neuronal marker NeuN in adult rats (16). An intraperitoneal injection of lipopolysaccharide (LPS) increases the number of GPNMB and OX42 double-positive cells in the area postrema (16). GPNMB is expressed in a subset of Iba1+ microglia in rat brain (27). GPNMB is highly expressed in BV2 mouse microglial cells after LPS treatment, significantly upregulates the expression of MMP-3, and GPNMB siRNA dramatically suppresses the expression of TNF-α, IL-1β, iNOS, and NO (28). Glioma-associated microglia strongly express GPNMB (29). CD11c+ microglia in APP/PS1 mice express high levels of GPNMB (30). Importantly, a subpopulation of Iba1-positive microglia that cluster around amyloid plaques expresses high levels of GPNMB in AD brains (20), consistent with our results.

Extensive transcriptome analysis of brains of AD mouse models identified a novel type of microglia termed as disease-associated microglia (DAM) or microglial neurodegenerative phenotype (MGnD) showing a unique transcriptional and functional signature (31,32). DAM is activated sequentially by TREM2-independent and TREM2-dependent pathways. GPNMB reflects a microglia activation state only present under neurodegenerative conditions, which is characterized by upregulation of a subset of genes, including TREM2, APOE, CLEC7A and CST7 (20,31,32). We validated an enhanced expression of
GPNMB on amoeboid and hypertrophic microglia accumulating in the frontal white matter of NHD brains and the frontal cortex of AD brains. GPNMB-expressing clusters of activated microglia resided on amyloid plaques of AD brains, where they were often in contact with APOE-immunoreactive plaques and phosphorylated tau-labeled neurofibrillary tangles. In contrast, neurons and reactive astrocytes did not consistently express GPNMB in NHD and AD brains. Thus, we conclude that the principal cell type expressing GPNMB in NHD and AD brains is microglia previously termed as DAM. However, we could not exclude the possibility that a subpopulation of oligodendrocytes in the white matter expresses GPNMB. These results indicate that the accumulation of GPNMB-immunoreactive microglia is a general phenomenon in neurodegenerative brains.

Several lines of evidence suggest that GPNMB plays a neuroprotective role. Tg mice overexpressing GPNMB show a smaller infarct volume compared with wild-type mice following brain ischemia-reperfusion injury (33). GPNMB Tg mice have an improvement in hippocampal memory tasks and long-term potentiation associated with increased levels of the AMPA receptor subunit GluA1 (26). GPNMB suppresses motor neuron cell death induced by mutant superoxide dismutase 1 (SOD1) or Tar DNA-binding protein 43 (TDP43), by activating the ERK1/2 and Akt pathways (18,19). GPNMB shows a neuroprotective effect on NSC-34 motor neurons by activating the phosphoinositide 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK)-extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK pathways via interacting with the alpha subunits of Na+/K+-ATPase (NKA) (34). Direct injection of a GPNMB expression plasmid into the gastrocnemius muscle of SOD1G93A mice increases the number of myofibers and prevents myofiber atrophy (35). Overexpression of GPNMB protects skeletal muscle from severe degeneration caused by long-term denervation in mice (36). All of these observations indicate a protective role of GPNMB against active degenerating processes. Therefore, the enhanced expression of GPNMB on microglia might play a protective role in development of NHD and AD brain lesions.

In conclusion, we identified enhanced GPNMB expression on microglia in the severely affected frontal white matter of NHD brains and the frontal cortex and the hippocampus of AD brains, suggesting that GPNMB-expressing microglia might play a neuroprotective role against ongoing extensive leukoencephalopathy and neurodegeneration.

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A potential significance of circ_0024169 down regulation in angiosarcoma tissue

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Summary
Circular RNAs (circRNAs) are recently characterized non-coding RNAs that have a closed continuous loop. CircRNAs might play important roles in the oncogenesis of several cancers. However, little is known about association between circRNAs and skin tumors. In this study, we tried to demonstrate the expression change of circ_0024169 in angiosarcoma, and to elucidate correlations between circ_0024169 expression in angiosarcoma tissues and clinical manifestation. RNA expression was evaluated by quantitative real-time PCR with TaqMan systems for circ_0024169 and linear isoform CUL5. Both relative circRNA levels (corrected for EEF1A1 levels) and circRNA levels/linear RNA expression ratio were evaluated. We found that both relative circ_0024169 levels and circ_0024169/CUL5 ratio was decreased in normal human dermal microvascular endothelial cells (HDMEC) and angiosarcoma cell line in vitro, compared to squamous cell carcinoma line. circ_0024169/CUL5 ratio was significantly reduced in angiosarcoma and pyogenic granuloma than other tumors in vivo, which were more evident than decreased relative circ_0024169 levels. On the other hand, relative circ_0024169 levels showed mild inverse correlation with the follow-up periods (duration between the first hospital visit and the last hospital visit/the date of death) of angiosarcoma patients. Taken together, circ_0024169/CUL5 ratio are likely to be useful as a diagnostic biomarker for vascular tumors, whereas circ_0024169 levels may have more potential as a prognostic marker of angiosarcoma. The future studies of the function of circRNAs may lead to the clarification of detailed mechanism of oncogenesis of angiosarcoma.

Keywords: Angiosarcoma, circular RNA, epigenetics

1. Introduction
Angiosarcoma is a rare malignant vascular tumor originating from endothelial cells of blood/lymphatic vessels. The tumor usually occurs in the skin, especially the scalp and face of elderly people. Its aggressive progression and resistance to standard chemotherapy result in frequent local recurrence and hematogenous metastasis to the lungs, pleura, or liver. The five-year survival rate of the patients is approximately 20-30% (1-3), and angiosarcoma is therefore known as one of the tumors with very poor prognosis. However, the etiology of this tumor is still unknown.

Circular RNAs (circRNAs) are non-coding RNAs characterized by its circularized shape. circRNAs have a closed continuous loop formed by back-splice reactions that involve a covalent junction between the 3'-and 5'-ends. This structure was firstly described in early 1990s and was initially considered as by-products of splicing errors (4,5). However, recent comprehensive analysis of

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transcripts by high-throughput sequencing demonstrated that circRNAs are widely expressed in human cells (6). A detailed analysis of the circRNA sequences revealed that some circRNAs contain a large number of target sequences of specific microRNAs (miRNAs): miRNAs are short RNA molecules consisting of about 22 nucleotides, which exhibit gene regulatory functions by silencing target translation via interaction with its 3’ untranslated region (3’ UTR). circRNAs are thought to bind specific miRNAs to its own sequence, and reduce intracellular active and free miRNA levels, thereby negatively regulate miRNA function. Such effects were firstly demonstrated using cultured cells (7), and then the phenomenon shown by the forced overexpression of circRNA coincided with that shown by the suppression of corresponding miRNA expression in zebrafish. Accordingly, circRNAs are thought to serve as ‘miRNA sponges’ (6-8). Furthermore, another important feature of circRNAs is increased resistance to RNase due to the lack of ends attacked by exonucleases (6).

Recently, circRNA expression was shown to be tissue/cell-specific (6,9), and are implicated in the tumorigenesis of several cancers (10-12). However, the role of circRNAs in skin tumors has not been examined. In this study, we tried to investigate the possibility that circRNAs may play roles in angiosarcoma, focusing on circ_0024169, which were reported to be dysregulated in oral cancer and colon cancer previously (13).

2. Materials and Methods

2.1. Patient materials

Skin specimens were obtained from five pyogenic granuloma, four malignant melanoma, three squamous cell carcinoma, seven normal subjects, and ten angiosarcoma. Seven control normal skin samples were from routinely discarded skin of healthy human subjects undergoing skin grafts. Institutional review board approval and written informed consent were obtained according to the Declaration of Helsinki before patients and healthy volunteers were entered into this study.

2.2. Cell culture

Adult human dermal microvascular endothelial cells (HDMEC) were obtained from Lonza (Walkersville, MD), and cultured according to the manufacturer’s recommendations (14). The angiosarcoma cell line, ISO-HAS, was isolated from a tumor tissue specimen, and was grown as previously described (15). A human cutaneous squamous cell carcinoma (SCC) cell line, A431, was obtained from ATCC (Manassas, VA), and were cultured in DMEM (Lonza) with 10% fetal bovine serum (Hyclone, Logan, UT) and Antibiotic-Antimycotic (Invitrogen, Carlsbad, CA) in a 5% CO2 incubator at 37°C (16).

2.3. RNA isolation

Total RNA isolation from cultured cells with RNeasy Mini Kit (Qiagen) and from paraffin-embedded sections with RNeasy FFPE kit (Qiagen, Valencia, CA) were performed according to the manufacturer’s instructions, as described previously (13). The quality of RNA was evaluated using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

2.4. RNase R digestion, cDNA synthesis, and quantitative real-time PCR

Total RNA samples were pooled, digested by DNase I (Epicentre, Illumina, San Diego, CA), and treated with RNase R (Epicentre) twice to remove linear RNAs and enrich circular RNAs in QIAzol Lysis Reagent (Qiagen) (13).

First-strand cDNA was synthesized using PrimeScript RT reagent Kit (Takara) from the total RNA and RNase R-treated RNA. PCR was performed on Thermal Cycler DiceTM Real Time System (TP800; Takara, Shiga, Japan). Quantitative real-time PCR with TaqMan systems was performed with primers and templates mixed with Premix Ex Taq (Probe qPCR, Takara). Primer and probes for hsa_circ_0024169 were from Takara. Primers for CUL5 and EEF1A1 were from Life Technologies, Thermo Fisher Scientific (Waltham, MA). DNA was amplified for 50 cycles of denaturation for 5 seconds at 95°C and annealing for 30 seconds at 60°C.

2.5. Statistical Analysis

Statistical analyses were carried out with Kruskal-Wallis test for the analysis of more than three groups, and Mann-Whitney tests were carried out for the comparison of medians between two groups. Correlations were evaluated by Pearson’s correlation coefficient. P-values < 0.05 were considered significant.

3. Results

3.1. circ_0024169 expression in angiosarcoma cell lines in vitro

We first examined the expression levels of circ_0024169 in angiosarcoma cell line ISO-HAS and HDMEC. SCC line A431 was also used for the disease control. TaqMan Gene Expression Assays were designed to distinguish between circ_0024169 and the corresponding linear isoform CUL5. Quantitative real-time PCR with TaqMan systems for the specific circular and linear isoform RNAs and for the housekeeping gene EEF1A1 was performed according to the previous report (13). Both relative circRNA levels corrected by housekeeping gene levels and circRNA levels/linear RNA expression ratio were evaluated.
As a result, relative circ_0024169 levels corrected by EEF1A1 levels were lower in endothelial cells (HDMEC and ISO-HAS) than in A431 in vitro, especially in HDMEC (Figure 1A). Furthermore, similar tendency was found in the circ_0024169/CUL5 ratio (Figure 1B).

3.2. circ_0024169 expression in angiosarcoma tissues in vivo

Then, the relative circ_0024169 levels and circ_0024169/CUL5 ratio in the tissue sections of normal skin (n = 7), angiosarcoma (n = 12), pyogenic granuloma (n = 5), malignant melanoma (n = 4), and SCC (n = 3) were determined with real-time PCR. There was no difference in the relative circ_0024169 levels between normal skin and angiosarcoma skin, although the levels tended to be increased in melanoma and SCC compared with normal skin (Figure 2A).

On the other hand, the circ_0024169/CUL5 ratio was highest in normal tissues than tumors. The values were not significantly different between normal skin and angiosarcoma skin, although the levels tended to be increased in melanoma and SCC compared with normal skin (Figure 2A).

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Figure 1. Expression of circ_0024169 in cultured HDMEC and ISO-HAS in vitro. The results of quantitative real-time PCR analyses to determine (A) the relative level of circ_0024169 (normalized to EEF1A1 levels) and (B) the expression ratio of circ_0024169 to its corresponding linear transcript (circ_0024169/CUL5) in cultured human dermal microvascular endothelial cells (HDMEC) and angiosarcoma cell line (ISO-HAS) were shown. The data in cultured SCC cell line (A431) was also shown as the disease control. The levels in HDMEC were set at 1.

Figure 2. Expression of circ_0024169 in vascular tumors in vitro. Relative circ_0024169 levels (A) and the circ_0024169/CUL5 ratio (B) in pyogenic granuloma (PG, n = 5), malignant melanoma (MM, n = 4), squamous cell carcinoma (SCC, n = 3), normal skin (NS, n = 7), and angiosarcoma (AS, n = 10) were shown. (C, D) Association between circ_0024169 expression and clinical features in angiosarcoma. (C) Correlation of relative circ_0024169 levels (normalized to EEF1A1 levels) with follow-up periods in patients with angiosarcoma was shown. (D) Correlation of circ_0024169/CUL5 ratio with follow-up periods was also presented. The minimum value in AS was set at 1. Bars show means. *p < 0.05.
lower in vascular tumors including pyogenic granuloma and angiosarcoma than in melanoma and SCC (Figure 2B). A significant difference was observed among these groups by the Kruskal-Wallis test (p = 0.0022). There were significant changes in the ratio between pyogenic granuloma and melanoma (p = 0.014), SCC (p = 0.025), or normal skin (p = 0.0045) by Mann-Whitney test. Also, the ratio of angiosarcoma was significantly decreased compared to that of melanoma (p = 0.040) or normal skin (p = 0.0068): The ratio was lower in pyogenic granuloma compared to angiosarcoma.

Taken together, the result indicated circ_0024169/CUL5 ratio tended to be reduced in vascular tumors, and seems to be more sensitive than relative levels to distinguish vascular tumors from others.

3.3. Correlations of circ_0024169 expression and clinical manifestation in patients with angiosarcoma

Lastly, we tried to determine the clinical significance of relative circ_0024169 levels or circ_0024169/CUL5 ratio in angiosarcoma patients. We found that relative circ_0024169 levels showed mild inverse correlation with the follow-up periods (duration between the first hospital visit and the last hospital visit/the date of death) (R = 0.28, Figure 2C). On the other hand, there was no correlation between the ratio and follow-up periods (R = -0.09, Figure 2D). Accordingly, relative circ_0024169 levels in angiosarcoma tissues may be more useful to predict prognosis than circ_0024169/CUL5 ratio. In Kaplan-Meier method of twelve patients divided into two groups (six patients with increased and decreased relative circ_0024169 levels or circ_0024169/CUL5 ratio), those with decreased relative circ_0024169 levels tended to show poorer prognosis (Figure 3A and 3B), but there was not statistically significant difference.

4. Discussion

In this study, we have presented two major findings. First, we found that both relative circ_0024169 levels and circ_0024169/CUL5 ratio was decreased in HDMEC and angiosarcoma cell line in vitro, especially in HDMEC.

On the other hand, circ_0024169/CUL5 ratio was significantly reduced in angiosarcoma and pyogenic granuloma in vivo, which were more evident than relative circ_0024169 levels. A remarkably decreased circ_0024169/CUL5 ratio in HDMEC in vitro may correspond to the reduced levels in pyogenic granuloma in vivo, because pyogenic granuloma is a benign and reactive vascular tumor of the skin. However, circ_0024169 levels showed a mild correlation with the follow-up period. Although there were not significant difference (p = 0.37), this might be because of small number of cases: Although circRNA experiments required a mass of RNA, we could not collect enough number of large samples to obtain adequate RNA. Larger study with increased number of patients will be needed in the future. Multiple in vitro experiments using various cell types including normal human keratinocytes, HaCaT cells, and human umbilical vein endothelial cells (HUVEC) should also be performed. There was no correlation between circ_0024169 / CUL5 ratio and follow-up periods.

Taken together, our pilot study with small patient number suggest the possibility that circ_0024169/CUL5 ratio are useful as a diagnostic biomarker for vascular tumors, whereas relative circ_0024169 levels may have more potential as a prognostic marker of angiosarcoma. Generally, both relative circRNA levels and circRNA levels/linear RNA ratio are important for the evaluation of circRNA expression, and each may have different clinical significance in human diseases: The back-splicing process is thought to be regulated by the ratio of circular to linear transcripts, and the relative abundance of differentially spliced circular isoforms is cell-type specific (17,18). For example, circRNA/linear RNA ratio negatively correlated with the cell proliferation, regardless of whether the linear RNA or circRNA alone was lower or higher expressed in tumor compared to normal tissue (13). However, there have been no studies showing their importance in skin tumors.

In this study, we focused on circ_0024169,
which were reported to be down-regulated in ovarian cancer and colon cancer (13). As described above, circRNAs are usually resistant to RNase treatment, and more stable than linear RNAs. Because the stable circRNAs tend to accumulate in non-proliferating cells while they are evenly distributed to daughter cells in proliferative cells, the circRNA levels are thought to become relatively low in tumor tissues (13). On the other hand, function of circRNAs in these cancers and angiosarcoma is still unknown. One of the important functions of circRNAs is to bind miRNAs as the sponge and inhibit their functions. miRNAs have already been implicated in the pathogenesis of angiosarcoma: For example, down-regulation of miR-210 stimulates cell proliferation via the induction of E2F3 and EphrinA3 in angiosarcoma (19). The expression of miR-214 and miR-126 are markedly elevated in the plasma of angiosarcoma patients (20). The identification of circ_0024169-associated miRNAs in angiosarcoma may resulted in the clarification of detailed mechanism of oncogenesis in angiosarcoma.

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Heart rate variability in a patient with alternating hemiplegia

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1. Introduction

Alternating hemiplegia of childhood (AHC) is a rare disorder characterized by repetitive episodes of transient hemiplegia. Although autonomic nervous system dysfunction is believed to be associated with AHC, there are no reports of heart rate variability (HRV) in patients with AHC. In the current study, we analyzed HRV in a 20-year-old female with this disorder. The frequency of paralytic attacks have decreased since the patient was a teenager, compared to when she was < ten years old; however, as a 20-year-old, she still experiences paralytic attacks several times per month to more than ten times per month. Thus far, she has only suffered paralytic attacks and no epileptic seizures. Using Sanger sequencing, Gly947Arg (2839G>A) in the sodium-potassium (Na⁺/K⁺)-ATPaseα3 subunit gene (ATP1A3) was confirmed from her blood sample. An elevated heart rate lasting one to two minutes and sometimes longer, was primarily observed at night while the patient was sleeping. Large fluctuations in HRV, including low- and high- frequency components, were primarily observed while the patient was sleeping but suppressed during paralytic attacks. These results confirm the presence of an autonomic nervous system disorder in AHC. Because large variation of the autonomic nervous function was observed at night, the pathophysiological function should be investigated for 24 hours.

Keywords: Transient hemiplegia, paralytic attack, autonomic nervous dysfunction, abnormal eye position

2. Case Report

Upward movement of the eyes and transient paralysis of the right or left upper limbs were noted at five months of age. Head control was possible at six months and sitting was possible at ten months. The paralytic attacks occurred at a frequency of once every day to once every three days. The patient was clinically diagnosed with AHC at a university hospital. The frequency of attacks have decreased since the patient...
was a teenager, compared to when she was < ten years old; however, as a 20-year-old, the patient still experiences paralytic attacks several times per month to more than ten times per month. The patient was once able to stand with support and cruise, but can now only sit and bear crawl and not cruise. Owing to intellectual disabilities, verbal communication is near impossible, but she can raise her hands when shown to do so. Complete assistance is required during mealtime, and she is unable to eat when attacks occur during this period. Thus far, she has only suffered paralytic attacks and no epileptic seizures. Anticonvulsant drugs such as sodium valproate, clonazepam and clobazam, have been administered. Flunarizine, which was privately bought by her mother, had been administered to her since the age of four. After coming to our institution at the age of 18, flunarizine administration was discontinued due to a lack of government insurance coverage, however, the frequency of attacks did not change after its cessation.

Using Sanger sequencing, Gly947Arg (2839G>A) in \textit{ATP1A3} gene was confirmed from her blood sample but her mother had no mutation in \textit{ATP1A3} gene. Approval of this case report with the gene analysis was indicated by her mother’s signature on the documents accepted by the ethics committee of our institutions.

Based on a 24-hour Holter electrocardiogram (ECG), the patient’s HRV was analyzed using software (MemCalc/Chiram 3 version 2.1.10, GMS) sixteen times for two years of her stay at our institution. This examination was completed during a paralytic attack three times out of sixteen examinations. Changes in RR intervals, heart rate, coefficients of variance of RR intervals (CVRR), low frequency (LF) [0.04-0.15Hz] and high frequency (HF) [0.15-0.4Hz] components of HRV and the compressed waveform of ECG, were analyzed.

An example showing changes in heart rate, CVRR, LF and HF components of HRV during a paralytic attack is depicted (Figure 1). Upward rotation of the eyes with conjunctival injection and lacrimation were noted at 14:40. The paralytic attack appeared at 14:50. When the examination began at 15:22, she was unresponsive to her name. At 20:10, she became responsive to her name, but her upper and lower limb paralysis continued. At 20:30, her eye position returned to normal and at 23:00 she could move her right hand but not her left. The paralytic

Figure 1. Changes in heart rate (HR), coefficient of variation of RR intervals (CVRR), low-frequency (LF) and high-frequency (HF) components. When this recording began at 15:22, she had paralytic attack as explained in the text. The upper two graphs show the changes that occur in eight beats of HR, averaged (beat/minute) and CVRR %. The red and blue graphs show HR and CVRR, respectively. The lower two graphs show the changes of the power components of LF and HF in heart rate variability (HRV). The light blue and dark blue graphs show LF and HF, respectively. X axis shows time from 15:22 to next day 8:40. CVRR, LF and HF were suppressed especially during the initial period of the paralytic attack from 15:22 to 16:30 (orange bar). Yellow arrows indicate HR elevation that continued for about one minute at 1:30 and 1:40. This is further demonstrated in Figure 2. Note the large amplitude fluctuations that occur in both LF and HF during sleep (shaded area from 0:45 to 5:15).
Figure 2. A compressed electrocardiogram (ECG) waveform. Note that transient tachycardia lasted for about one minute at 1:30 am and 1:40 am. This corresponds to yellow arrows shown in Figure 1.

Figure 3. Changes in HR, CVRR, LF and HF components recorded when there was no paralytic attack. Green arrows indicate HR elevation lasting ten minutes and thirty minutes with CVRR reduction during sleep (shaded area). Although LF and HF variations were large at night as in Figure 1, they were relatively weak during the HR elevations. Compared to reduction of CVRR, LF and HF recognized in the initial period of paralytic attack in Figure 1, such reduction was not observed in the same time period.
attack had completely ceased by 0:00 of the next day. One hour after the beginning of the examination, CVRR, LF and HF components of the HRV were greatly suppressed (Figure 1).

The suppression of CVRR, LF and HF components of the HRV that occurred during the initial period of the attack was confirmed in the other two examinations. An elevated heart rate lasting one to two minutes and sometimes longer, was primarily observed at night while the patient was sleeping. The elevated heart rates that lasted roughly one minute were demonstrated using the Holter ECG (Figure 2). The amplitude and variation of LF and HF components were also higher while the patient slept (Figure 1 and Figure 3). Except for the heart rate elevation lasting one or two minutes, heart rate elevation with reduced CVRR lasting five to forty minutes were observed two or three times during sleep in most recordings. An example of the recording is shown with green arrows in Figure 3.

3. Discussion

After the first description of AHC (8), the characteristic clinical manifestations have been the mainstay of diagnostic criteria for AHC (9). The current case has consistently shown these clinical manifestations from the first paralytic attack to the present day. AHC has been associated with an increased risk of sudden death that may be caused by lethal cardiac arrhythmias (10). Cardiac dysfunction may account for some of the unexplained premature mortality of patients with AHC (6).

HRV may be used to assess autonomic imbalances, diseases, and mortality. HF power primarily reflects a parasympathetic influence, while LF power has been shown to reflect both sympathetic and parasympathetic influences (11). Epilepsy is associated with reduced HRV (12). In the present study, both LF and HF components were low during the initial period of the paralytic attacks. It has been reported that sleep relieves paralytic attacks in patients with AHC, however, the present study revealed a large amplitude and variation of LF and HF components, or autonomic dysfunction, during sleep. Recently sleep dysfunction with abnormal apnea-hypopnea index and mean arousal index was reported in patients with AHC (13). Sleep disturbance related to AHC should be studied further.

In conclusion, analysis of HRV revealed autonomic nervous dysfunction in a patient with AHC. Because large variation of the autonomic nervous function was observed at night, the biological rhythm should be investigated for 24 hours.

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References


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Tumefactive fibroinflammatory lesion successfully treated with Rituximab

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1. Introduction

Tumefactive fibroinflammatory lesions (TFIL) are a subset of inflammatory pseudotumors most commonly located in the head and neck that are characterized as a rare, locally destructive mass with benign findings on histopathology. Epidemiological data is limited with disease information restricted to case reports and case series from published materials (1-2). The historical use of various names in the medical literature further complicates our understanding of this diagnosis (3). Although there are no standardized treatment guidelines, therapy for TFIL typically includes a combination of surgical resection and steroids. Many patients experience recurrence once steroids are tapered, leading practitioners to explore alternative therapeutic options including immunomodulators (1-5).

Herein we present a case of TFIL of the infratemporal region successfully treated with rituximab following a failed response to steroids.

2. Case Report

A 45-year-old female presented with otorrhea and a right middle ear effusion for two months. The symptoms persisted despite placement of a pressure equalization tube. Five months later, she developed CN V₃ pain/numbness, trismus, headache, and autophony. MRI showed a diffuse infiltrative mass in the right infratemporal region involving the trigeminal ganglion. Biopsy revealed benign fibromuscular and adipose tissue with lymphoplasmacytic infiltrate, giving a diagnosis of TFIL. Resection would be very difficult given tumor location. Initial treatment included an extended course of steroids without response, and interval disease progression. Two courses of rituximab 375 mg/m² weekly × 4 given 3 months apart were then completed with excellent tolerance. With sixteen months following induction, the patient reports minimal symptoms with radiographic findings confirming continued disease regression. Rituximab is a potential treatment option for patients with TFIL without response to steroids.

Keywords: Rituximab, tumefactive fibroinflammatory lesion, pseudotumor, immunomodulators, inflammatory pseudotumors

Summary

Skull base pseudotumors, or tumefactive fibroinflammatory lesions (TFIL), are tumors characterized by local destruction with benign histopathology. Treatment includes surgery and steroids with varying degrees of symptom relief. A 45-year-old female presented with right otorrhea and middle ear effusion, which progressed to CN V₃ pain/numbness, trismus, headache, and autophony. MRI showed a diffuse infiltrating mass in the right infratemporal region involving the trigeminal ganglion. Biopsy revealed benign fibromuscular and adipose tissue with lymphoplasmacytic infiltrate, giving a diagnosis of TFIL. Resection would be very difficult given tumor location. Initial treatment included an extended course of steroids without response, and interval disease progression. Two courses of rituximab 375 mg/m² weekly × 4 given 3 months apart were then completed with excellent tolerance. With sixteen months following induction, the patient reports minimal symptoms with radiographic findings confirming continued disease regression. Rituximab is a potential treatment option for patients with TFIL without response to steroids.

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image guidance and mucosal graft of the defect. Pathology revealed small lymphoid cells and plasma cells without atypia intermixed with hypocellular bands of fibrosis and entrapped atrophic skeletal muscle (Figure 2). In light of these pathological findings, she was diagnosed with an inflammatory pseudotumor, specifically a TFIL. A high dose prednisone taper was initiated at that time, which provided resolution of the headache and improved V3 sensation and pain in the immediate postoperative period. She was referred to a hematologist with experience treating similar lesions. Her symptoms returned within three weeks of her admission.

Figure 1. T1 MRI pre and post treatment. (A), Pre-treatment contrasted coronal T1 MRI showing diffuse, enhancing, infiltrative mass involving the infratemporal fossa, oropharynx, and soft palate, extending into the foramen ovale with displacement of the trigeminal ganglion, in addition to questionable involvement of the right facial nerve and right vidian canal. (B), Contrastened coronal T1 MRI seven months after completion two cycles of rituximab, demonstrating significantly less enhancement in the infratemporal fossa.

Figure 2. Histopathology report. (A), Morphologic examination of the biopsy revealed hypocellular bands of fibrosis entrapping skeletal muscle with atrophic change. (Hematoxylin and Eosin stain; original magnification 200×). (B), In areas, lymphoid aggregates were seen, comprised of small lymphoid cells without atypia. (Hematoxylin and Eosin stain; original magnification 200×). (C), Immunohistochemical analysis revealed predominance of CD3 positive T-cells within lymphoid aggregates. (CD3 immunohistochemical stain; original magnification 200×). (D), Immunohistochemical analysis revealed few CD20 positive B-cells within lymphoid aggregates. (CD20 immunohistochemical stain; original magnification 200×).
operation while tapering the steroid dose, requiring an increase and extension of the steroid taper. She reported improvement of her symptoms with the higher steroid dosage.

Three months into the slow steroid taper, she presented with a cushingoid appearance, worsened headaches, CN V₁ pain/numbness, autophony, and a new right sided House-Brackmann 3/6 facial palsy. Repeat MRI at that time revealed interval progression of the infiltrative process with more conspicuous enhancement at the foramen ovale and extension of disease along the lateral aspect of the right cavernous sinus tracking posteriorly along the greater superficial petrosal nerve to the level of the geniculate ganglion. Due to the progression of disease, a second endoscopic biopsy was performed again confirming TFIL without evidence of malignancy. At that time, alternate therapies were explored, including rituximab, due to positivity for CD20 within a subset of the lymphoid cells. The patient was treated with rituximab monotherapy consisting of 375 mg/m² weekly × 4 treatments. She completed her steroid taper prior to her 3rd dose of rituximab. The MRI two months later demonstrated significant radiologic improvement with less conspicuous enhancing soft tissue fullness in the infratemporal fossa. She had ongoing improvement in symptoms and proceeded to a second course of rituximab monotherapy three months following the first cycle.

The patient tolerated her rituximab treatments without adverse events. A MRI seven months following her second course of rituximab demonstrated significant improvement with much less enhancement in the infratemporal fossa (Figure 1B). At her most recent follow up one year after completing treatment, she reports feeling very well with only faint right sided facial numbness, rare headaches in the right temple, and occasional trigeminal pain controlled with over-the-counter non-steroidal anti-inflammatory medications. Her facial paresis has completely resolved, and she notes much less autophony. No further corticosteroids have been required, and her Cushingoid appearance has resolved. A repeat MRI at that time shows further regression of disease.

3. Discussion

Inflammatory pseudotumors (IP) are benign, fibrosclerosing lesions found in many organ systems including lung, gastrointestinal tract, genitourinary tract, hepatobiliary, orbit, brain, skull base, and soft tissues of the trunk and extremities, with the most common sites being lung, orbit, nasopharynx, inner ear, and skull base. Pseudotumors are found across a broad age range from newborns to patients in their 80s, with the 3rd and 4th decade of life being most common (1). Pseudotumors are rare and difficult to classify given the lack of clear diagnostic criteria. Prior nomenclature has included plasma cell granulomas, xanthogranulomas, or histiocytomas. Subclasses of IP include TFIL, inflammatory myofibroblastic tumor (IMT), and IgG4 related disease.

Tumefactive fibroinflammatory lesion was first described in 1975 as sclerosing cervicitis and the term TFIL was first used in 1983. TFIL is characterized by an aggressive, locally destructive lesion, usually of the head and neck region, resembling neither malignancy nor infection (4). Lesions can invade adjacent soft tissues, muscles, and neurovascular structures, and can also erode bone, leading to meningeal and CNS involvement (2). Clinical symptoms are site dependent and tend to be rapidly progressive with headache, otalgia, vision changes, and hearing loss being among the most common complaints at presentation (1). In up to 20% of cases, mediastinal or retroperitoneal fibrosclerotic lesions, orbital pseudotumor, or Riedel's thyroiditis may be found on the staging evaluation (5). While the etiology of the disease is unknown, proposed mechanisms include an exaggerated response to chronic infection or an autoimmune reaction to a previous viral infection (4). Histopathology shows sclerotic fibrous inflammatory tissue with plasma cells and lymphocytes without cytologic atypia, necrosis, significant mitotic activity, or pleomorphism that would be seen in malignant counterparts such as fibrosarcomas (1,6). TFIL lacks significant numbers of IgG4 positive plasma cells and storiform pattern of fibrosis as seen in IgG4 related disease. TFIL lacks the ALK positivity and intranuclear eosinophilic inclusions as seen in IMT (7-9).

No standardized guidelines for treatment for IP and TFIL are available given the rarity of the diagnosis. Treatment is guided by the location and severity of disease. Past treatments have included combinations of steroids, surgical resection, radiation, and/or immunomodulators. Treatment with immunomodulators have included cyclophosphamide, mycophenolate mofetil, methotrexate, alpha interferon, azathioprine, and rituximab (1,2,4,8-13). Complete resection is preferred; however, tumor location may prevent a safe and tolerable approach (2). Lesions generally demonstrate good initial response to steroids, but many patients experience recurrence as the steroid is tapered, as seen in our patient. A meta-analysis of inflammatory pseudotumors of the skull base by Alyono et al. reported that 76% of patients had no recurrence or progression after the first course of steroids with without partial resection. However, only 33% of patients had complete symptom resolution, and even less had radiologic resolution (1). A separate case series cited the recurrence rate > 20% for head and neck cases, with only 40-50% of patients achieving complete remission (9).

Rituximab has recently emerged as a successful treatment option for patients who have failed prior surgical and steroid treatment. While the exact mechanism of rituximab is not clear, the tumor response
is likely due to induction of apoptosis secondary to antibody dependent, cell-mediated toxicity, and complement activation (9). Several case studies have shown successful use of rituximab for inflammatory pseudotumors in the jaw (8) and temporal bone (9) in addition to IgG4-related disease in the orbit (10-11,13-14) and temporal bone (15). No reports to date have used rituximab for successful treatment of TFIL specifically. Reported dosing regimens of rituximab have included 1,000 mg Q2wk × 2 doses versus 4 weekly doses at 375 mg/m² (8,13-15). The weekly schedule was chosen for our patient based on experience treating indolent non-Hodgkin B-cell lymphomas and other non-malignant inflammatory diseases like vasculitis, graft-versus-host disease, immune thrombocytopenia, and pemphigus vulgaris.

The most common side effect of rituximab is an infusion reaction consisting of urticaria, fever, chills, angioedema, and hypotension, occurring in about 18% of patients, but rarely severe. Reversible myelosuppression occurs in 2-4% of patients, but increases in frequency when combined with chemotherapy (16). However, effects of long term steroid use include osteoporosis, adrenal insufficiency, hyperlipidemia, and hyperglycemia, which increase in severity with increasing dose (17). High dose steroid regimens have been found to cause adverse effects in up to 33% of patients, even with short term treatments (18). Similarly, the incidence corticosteroid-induced lipodystrophy has been found to be 65% in patients on daily steroid therapy for 6 months (19). The frequency and severity of these adverse effects suggests therapy with rituximab could be a promising alternative.

Our patient continues to show symptomatic and radiographic improvement now sixteen months out from initiation of treatment with rituximab. Our report suggests that for patients with TFIL who have an unresectable lesion and/or have failed treatment with steroids, rituximab has the potential to be an effective steroid-sparing treatment option. We propose that more research is needed to explore the role of rituximab in the treatment of all classes of inflammatory pseudotumors.

References


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A novel mutation in TTN gene in a Saudi patient with bilateral facial weakness and scapular winging

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Summary

Titin (TTN) is a large gene with 363 exons that encodes a large abundant protein (longest known polypeptide in nature) that is expressed in cardiac and skeletal muscles. TTN has an important role in the sarcomere organization, assembly of muscles, transmission of the force at the Z-line, passive myocyte stiffness, and resting tension maintenance in the I-band region. Mutation in extreme C terminus of TTN, situated at the end of M-band of the TTN in chromosome 2q31, results in tibial muscular dystrophy (TMD), also called Udd Distal Myopathy, which is an autosomal dominant distal myopathy. In this article, we report a novel mutation in TTN gene in a Saudi patient with bilateral facial weakness and scapular winging. This report adds to the literature a heterozygous missense variant c.85652C>G, p.(Pro28551Arg) in TTN gene, which may be related to genes that cause the disease, but more case validation is needed. The novel mutation described in the present study widened the genetic spectrum of TTN-associated diseases, which may benefit studies addressing this disease in the future.

Keywords: Titin, TTN, tibial muscular dystrophy, neuromuscular disorders, Saudi Arabia

1. Introduction

Titin (TTN) is a large gene with 363 exons that encodes a large abundant protein (longest known polypeptide in nature) that is expressed in cardiac and skeletal muscles. TTN protein has an important role in sarcomere organization, assembly of muscles, transmission of the force at the Z-line, passive myocyte stiffness, and resting tension maintenance in the I-band region (1). Mutation in extreme C terminus of TTN, situated at the end of M-band in chromosome 2q31, results in tibial muscular dystrophy (TMD, MIM#600334), also called Udd Distal Myopathy. In addition, truncation mutations of TTN are considered the most common cause of familial dilated cardiomyopathy (2). TMD is a mild autosomal dominant distal myopathy involving the anterior compartment muscles of the lower legs (3). TMD was first described in Finnish patients; its prevalence in Finland is estimated to be > 1/10,000. TMD usually begins in adulthood, between ages 35-55 years, and is a slowly progressive, benign distal myopathy typically restricted to the anterior muscles of the lower leg (tibialis anterior, extensor hallucis longus, extensor digitorum longus). In some patients, muscle weakness also progresses to proximal lower leg musculature (4).

In this article, we report a novel mutation in TTN gene in a Saudi patient with bilateral facial weakness and scapular winging. What makes this report interesting is not only the novel genetic mutation but also the atypical clinical presentation with lack of distal muscle dystrophy (TMD, MIM#600334), also called Udd Distal Myopathy. In addition, truncation mutations of TTN are considered the most common cause of familial dilated cardiomyopathy (2). TMD is a mild autosomal dominant distal myopathy involving the anterior compartment muscles of the lower legs (3). TMD was first described in Finnish patients; its prevalence in Finland is estimated to be > 1/10,000. TMD usually begins in adulthood, between ages 35-55 years, and is a slowly progressive, benign distal myopathy typically restricted to the anterior muscles of the lower leg (tibialis anterior, extensor hallucis longus, extensor digitorum longus). In some patients, muscle weakness also progresses to proximal lower leg musculature (4).

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involvement, which is the characteristic mark of this genetic disorder.

2. Case Report

A proband III-1, a 34-year-old male presented to the neurology clinic with weakness of the upper extremities and difficulty in performing activities of daily living such as combing his hair and using utensils. He denied swallowing difficulty, visual symptoms, sensory symptoms, cramps, fasciculations, headache, skin lesions, joint symptoms, or oro-genital ulcers. Systemic review and past history were unremarkable. He denied any history of diabetes mellitus, heart disease, malignancy, or connective tissue disorders. Family history was remarkable for a similar disease in all of his three brothers. Parents of the patient are not consanguineous.

His vital signs and general medical examination were normal. Neurological examination showed normal higher mental functions, speech, and cranial nerves II to XII. Motor examination showed winging of the left scapular to medial side and mild facial diplegia. Lower limb examination including tone, power, and reflexes were normal. Coordination and cerebellar functions were normal. Serum creatine kinase (CK) level was elevated at 423 IU/L (normal 27-132 IU/L), CK-MB isoenzyme at 12.9 IU/L (normal 5-25 IU/L), and CK-MB percentage of 3.05% (normal 3-5%). The rest of the blood tests were unremarkable.

Nerve conduction studies were normal. Electrocardiogram and echocardiography were normal. Needle electromyography was performed on the serratus anterior, latissimus dorsi, deltoid, rhomboids major, and rhomboids minor. Spontaneous denervation potentials were not observed. The muscle membrane was stable with normal insertional activity. The motor units in the rhomboids major and minor were relatively large and long with reduced recruitment. The serratus anterior was difficult to sample, likely due to atrophy. The MUAPs in the latissimus dorsi appeared relatively small with relatively shorter duration than the neighboring muscles. The electromyography of the deltoid was unremarkable. These findings reflect a long-standing chronic myopathic process involving the shoulder girdle muscles on the left side, especially the rhomboids and latissimus dorsi with a dropout of motor units due to the chronicity of the myopathic process.

The detailed family pedigree was drawn after getting the detailed information from the parents as shown in figure 1. Given the strong family history, genetic testing was performed using whole exome sequencing. TTN gene mutation identified through exome sequencing, which was further validated by using Sanger sequencing in all members III-1, III-2, IV-1, IV-2, IV-3, IV-4, IV-5 of the family. Primers were designed for the target region, and TTN gene was amplified by polymerase chain reaction (PCR). Purified PCR products were Sanger sequenced by the Big Dye terminator method by using Applied Biosystems 3700 (ABI 3700).

We identified a heterozygous missense variant c.85652C>G, p.(Pro28551Arg) in TTN gene. The variant affects six RefSeq annotated transcripts and is located in exon 326 in the metatranscript (NM_001267550.1) containing a total of 363 exons. The variant has not been observed in large reference population cohorts. The variant is predicted damaging by in silico pathogenicity prediction tools. Furthermore, in the Greater Middle East (GME) variome minor allele frequency was 0.00 in the database. Moreover, PolyPhen 0.7, and SIFT 0.12, PhyloP and (phyloP46way_placental) predicted as a causative mutation. This mutation was absent in the Human Gene Mutation Database (HGMD, www.hgmd.cf.ac.uk/) MIM. 1000 genome (http://www.internationalgenome.org/) and ExAc (Version 0.3.1)

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Figure 1. A large consanguineous family from Saudi Arabia showing the disease phenotype. The samples marked with asterisks were available for genetic testing. Blocked square and circles indicate the infected individuals. Mutant (m) and wild-type (wt) allele are marked in the analyzed individuals.
for the truncating French TMD mutation c.107890C>T (p.Q35964*; NM_001267550.1) has also been reported with a proximal phenotype somewhat different from the homozygous Finnish LGMD2J patients. In the French patient, the disease onset was at 25 years, and the first muscle weakness occurred in the proximal upper limbs. Weakness and wasting progressed to all four limbs, and the patient lost ambulation at the age of 56 years. In addition, in consanguineous Chinese Han pedigree with autosomal recessive LGMD, a homozygous missense mutation TTN c.107788T>C, p.(Trp35930Arg) cosegregated with the disorder in the family and was absent from the ExAC control cohort.

TMD FINmaj mutation is predicted to cause cleavage of a larger part of TTN C-terminus, because immunofluorescence studies of homozygote LGMD2J muscles showed an absence of titin M8/M9 domain epitopes. Also, Western blot analysis showed a severe reduction of the C-terminal TTN fragments in LGMD2J patient muscle. Loss of protein interactions of C-terminal TTN is thus likely consequence of the TMD/LGMD2J mutations. Using yeast 2-hybrid analysis, Sarparanta et al. found that TTN containing FINmaj mutation failed to interact with myospryn. Ceyhan-Birsoy et al. identified five individuals with childhood-onset centronuclear myopathy (CNM) who had compound heterozygous mutations in TTN. The patients presented with diffuse weakness, respiratory problems, occasional feeding difficulties and most had scoliosis and hypotonia. In one patient, both the maternally and paternally inherited mutation was a truncating change. Another patient was a compound heterozygote for a nonsense variant c.77989C>T (ex326) and a frameshift c.108114delA (ex326). Another patient, a 5-year-old boy, had also two radical mutations inherited one from each parent, splice site mutation c.15721+1G>A and two frameshifts c.44998_45001del (ex358). None of the five patients had a noteworthy cardiac phenotype, although the ages at last examination only ranged from 5 to 19 years.

Muscle MRI is a very useful tool in patients for the truncating French TMD mutation c.107890C>T (p.Q35964*; NM_001267550.1) has also been reported with a proximal phenotype somewhat different from the homozygous Finnish LGMD2J patients. In the French patient, the disease onset was at 25 years, and the first muscle weakness occurred in the proximal upper limbs. Weakness and wasting progressed to all four limbs, and the patient lost ambulation at the age of 56 years. In addition, in consanguineous Chinese Han pedigree with autosomal recessive LGMD, a homozygous missense mutation TTN c.107788T>C, p.(Trp35930Arg) cosegregated with the disorder in the family and was absent from the ExAC control cohort. TMD FINmaj mutation is predicted to cause cleavage of a larger part of TTN C-terminus, because immunofluorescence studies of homozygote LGMD2J muscles showed an absence of titin M8/M9 domain epitopes. Also, Western blot analysis showed a severe reduction of the C-terminal TTN fragments in LGMD2J patient muscle. Loss of protein interactions of C-terminal TTN is thus likely consequence of the TMD/LGMD2J mutations. Using yeast 2-hybrid analysis, Sarparanta et al. found that TTN containing FINmaj mutation failed to interact with myospryn.

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with TMD and usually shows typical disease pattern characterized by dystrophic changes in the muscles of the anterior compartments of the lower legs starting in the anterior tibial muscle. Serum CK values are normal-mildly elevated, and there is no associated cardiomyopathy. The final diagnosis is confirmed by a genetic analysis of TTN gene (3). Next generation sequencing methods are extremely useful in the detection of genetic mutations in TMD, which are usually novel mutations and challenging to interpret. In silico prediction tools require a careful way of interpretation including functional studies, which are unfortunately not feasible with TTN due to its huge size that prevents the cloning and expression of the full-length protein in *in vitro* systems (5).

In this study, we identified a heterozygous missense mutation in TTN gene c.85652C>G, p.(Pro28551Arg) in a Saudi patient with bilateral facial weakness and scapular winging. This novel mutation in the TTN gene was suggested to be the genetic cause of the disease and further expanded the genetic spectrum of TTN-associated diseases in this family. This report adds to the literature a novel variant in TTN gene, which may be related to genes that cause the disease, but more case validation is needed. The novel mutation described in the present study widened the genetic spectrum of TTN-associated diseases, which may benefit studies addressing this disease in the future.

**References**


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Hypogenesis of right hepatic lobe in a laparoscopic cholecystectomy for acute gallstone cholecystitis: A case report

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Summary
Hypogenesis or agenesis of right hepatic lobe is a rare abnormality and is generally associated with gallbladder and biliary tract abnormalities. Cases of biliary injury following cholecystectomy have been reported in patients with agenesis of right hepatic lobe because the anatomical anomalies complicate the surgical approach. We report a case of laparoscopic cholecystectomy in a patient with hypogenesis of right hepatic lobe. A 92-year-old male patient was admitted to our hospital with fever and right lower abdominal pain with suspected acute appendicitis. Abdominal computed tomography revealed gallstones with acute cholecystitis and hypogenesis of right hepatic lobe. He underwent laparoscopic cholecystectomy with the left semilateral decubitus position. The patient’s postoperative course was uneventful. In conclusions, some patients with liver lobe hypoplasia do not present with the typical symptoms of acute cholecystitis due to dislocation of the gallbladder. The left semilateral decubitus position with modified placement of port sites is useful for laparoscopic cholecystectomy in patients with hypogenesis of right hepatic lobe.

Keywords: Hypogenesis of right hepatic lobe, acute cholecystitis, laparoscopic cholecystectomy, agenesis of right hepatic lobe

1. Introduction
Hypogenesis or agenesis of right hepatic lobe is a rare abnormality and is generally associated with anatomical variations of the gallbladder, biliary tract, and neighboring organs (1-4), including absence of the right portal vein and right intrahepatic bile duct. The incidence of lobar aplasia or hypoplasia is 0.005% in autopsy studies (5,6). Only 65 cases of hypogenesis or agenesis of right hepatic lobe have been reported until 2018 (5,7). Although patients with hypogenesis of right hepatic lobe are generally asymptomatic, some exhibit portal hypertension and liver cirrhosis (4).

The tender point in acute cholecystitis is typically located in the right subcostal or epigastric region due to the normal location of the gallbladder. However, if this location varies, it may confound the evaluation of symptoms. Imaging tests like ultrasonography (US) and computed tomography (CT) are helpful for the diagnosis of cholecystitis and for anatomical evaluation prior to surgery.

Laparoscopic cholecystectomy was developed over 30 years ago and is currently the gold standard for cholecystectomy (8). Because anatomical abnormalities complicate the procedure, there are some reports of biliary injury during cholecystectomy in patients with congenital anatomical variations, including agenesis of right hepatic lobe (2,3,9). There is a case report of laparoscopic cholecystectomy in a patient with agenesis of right hepatic lobe (10). However, an
accurate diagnosis was not possible before the surgery in that case, because the patient did not undergo CT or magnetic resonance imaging (MRI) before the surgery. After the operation, the patient was diagnosed with agenesis of the right hepatic lobe in that report. Herein, we report a patient with gallstone cholecystitis and a preoperative diagnosis of hypogenesis of right hepatic lobe who underwent uneventful laparoscopic cholecystectomy.

2. Case Report

A 92-year-old man presented to a clinic with fever and tenderness of the right lower abdomen; he was suspected of having acute appendicitis and was referred to our emergency room. His medical history included dementia, osteoarthritis of the knee, and hypertension, with no previous hepatobiliary conditions. He had never injured his abdomen or undergone surgery. Body temperature was 37.6°C.

The initial laboratory evaluation revealed a total bilirubin level of 2.2 mg/dL (normal 0.3-1.4 mg/dL), direct bilirubin 0.6 mg/dL (normal 0-0.5 mg/dL), aspartate aminotransferase 24 U/L (normal 10-35 U/L), alanine aminotransferase 22 U/L (normal 5-35 U/L), gamma glutamyl transferase 18 U/L (normal 5-60 U/L), alkaline phosphatase 212 U/L (normal 102-302 U/L), lactate dehydrogenase 225 U/L, (normal 100-250 U/L), serum albumin 3.3 mg/dL (normal 4.0-5.3 mg/dL), white blood cell count 9.9 K/mm³ (normal 3.9-9.8 K/mm³), hemoglobin 13.1 g/dL (normal 13.5-17.6 g/dL), hematocrit 37.2% (normal 39.8-51.8%), platelet count 125 K/mm³ (normal 131-362 K/mm³), (100-250), and C-reactive protein 12.3 mg/dL (normal 0.0-0.3 mg/dL).

The gallbladder could not be identified during an US through the abdominal wall, but an abdominal CT for screening of abnormalities revealed gallstones and signs of acute cholecystitis, such as pericholecystic fat stranding. In addition, the gallbladder was shown to be located on the right dorsal side of the liver, which was missing the right lobe (Figure 1A). The right portal vein was not visualized in the contrast-enhanced CT (Figure 1B). Two hepatic arteries originated from the superior mesenteric artery and perfused the medial side of the liver, and the cystic artery branched out from one of them. There was no right hepatic vein (Figure 1C). Magnetic resonance imaging (MRI) for visualization of the pancreaticobiliary system showed multiple hepatorenal cysts and absence of the right intrahepatic bile duct (Figure 1D).

The patient was diagnosed with acute cholecystitis due to gallstones and hypogenesis of right hepatic lobe. However, percutaneous transhepatic gallbladder drainage was difficult for the patient due to dislocation of the gallbladder. He was hospitalized and underwent conservative treatment for 9 days until the scheduled

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Figure 1. A-C Contrast-enhanced CT. (A) The gallbladder was located on the right dorsal side of the liver, which had no right lobe; (B) The right portal vein was not visualized; (C) There were several veins draining the medial side, but no right hepatic vein; (D) MRI cholangiography showed no right hepatic duct.
of their condition, which makes acute cholecystitis in these patients difficult to diagnose based on symptoms alone. Therefore, whenever a case of hypogenesis of right hepatic lobe is discovered incidentally, the patient should be informed of their condition.

There is a previous case report of laparoscopic cholecystectomy in a patient with agenesis of right hepatic lobe (10); however, in that case, the patient did not have a preoperative agenesis of right hepatic lobe. Therefore, the position of the patient and the location of the ports during that surgery were not modified. The patient with hypogenesis of right hepatic lobe has a gallbladder on the dorsal side of the liver compared with normal patients. Therefore, laparoscopic cholecystectomy for them with normal position and normal port sites is expected to be more difficult than that with normal anatomy. We searched PubMed for English-written articles that mention hypogenesis or agenesis of right hepatic lobe. To our knowledge, until the time of this writing in March 2019, this is the first report of laparoscopic cholecystectomy in a patient with a preoperative diagnosis of hypogenesis of right hepatic lobe. Due to improvements in diagnostic imaging, increasing numbers of patients with a preoperative diagnosis of hypogenesis or agenesis of right hepatic lobe are expected. We chose the left semilateral decubitus position and shifted the four ports approximately 3 cm to the right, and these modifications were helpful in the safe completion of the procedure. However, the most appropriate position and port sites should be based on the preoperative evaluation and selected according to the anatomical variations in each patient.

There have been some previous reports of biliary injury during cholecystectomy in patients with agenesis of right hepatic lobe (2,3,9), and conversion to open surgery is required in some cases to avoid such injury.
(9). However, the laparoscopic approach has the advantage of a clearer operative field of view compared with laparotomy and may provide better visualization of the right retrohepatic area. Conversely, open surgery may require a large incision and mobilization of the liver in patients with hypogenesis of right hepatic lobe due to the location of the gallbladder.

Here we present a case of hypogenesis of right hepatic lobe in a patient who underwent laparoscopic cholecystectomy for acute cholecystitis. We conclude that in the present study, due to the retrohepatic location of the gallbladder, the left semilateral decubitus position and modification of the port sites were advantageous for successful completion of the procedure.

References


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Splice receptor-site mutation c.697-2A>G of the COL1A1 gene in a Chinese family with osteogenesis imperfecta

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Summary

Osteogenesis imperfecta (OI) is a genetic disorder characterized by bone fragility and blue sclerae, which are mainly caused by a mutation of the COL1A1 or COL1A2 genes that encode type I procollagen. Mutations in the splice site of type I collagen genes are one of the mutations that cause OI and usually lead to a mild or moderate OI phenotype. A heterozygous A to G point mutation in intron 9 at the -2 position of the splice receptor site of COL1A1 was identified in a family with type I or IV OI. Three affected individuals in four generations of one family all presented with several clinical symptoms. They all had pectus carinatum, flat feet, gray-blue sclerae, and normal stature, teeth, hearing, and vision. Forearm fractures, small joint dislocations, and muscle weakness were all present in the patient’s father and grandmother, who presented with a moderate type IV phenotype. The 10-year-old proband with type I OI had suffered a fracture twice, but had no history of joint dislocation or skin hyperextensibility. Charting the family helped to identify clinical symptoms in patients with mutations at the N-terminal of type I collagen genes.

Keywords: Osteogenesis imperfecta, splice receptor-site mutation, COL1A1, N-terminal of type I collagen

1. Introduction

Osteogenesis imperfecta (OI), also called brittle bone disease, is a genetic connective tissue disorder with a broad phenotypic variation and genetic heterozygosity. Autosomal dominant mutations in COL1A1 and COL1A2 are the main cause of classic osteogenesis imperfecta, which is characterized by bone fragility, blue sclerae, defects in the teeth, and deficits in hearing and vision (1–3). Epidemiological data on OI varies greatly, with an average prevalence of 1 in 15,000 to 20,000 births worldwide (4). The proportion of OI and its subtypes differs markedly among certain races or ethnicities, the ratio of OI type I to OI type III is seven to one in white Australians (5). In North America, the number of black patients with type III OI is six times that of black patients with type I OI (6). A total of 3,548 patients with OI have been registered (http://oi.gene.le.ac.uk). Epidemiological data on Chinese patients with OI have not been available until now.

A splice site mutation occurs in the processing of pre-mRNA into mature mRNA and can lead to a substitution, insertion, deletion, or frameshift. Mutations in either splice-donor or splice-acceptor sequences may lead to retention of intronic DNA or exon skipping, hence the production of abnormal
proteins (7). Over 300 splice site mutations have been
documented in the OI mutation database; most affect
the COL1A1 gene and most are substitutions (8,9).

The type I collagen gene is also the gene responsible
for other genetic bone diseases including Caffey disease
(10), arthrochalasias Ehlers-Danlos syndrome (aEDS) (11-
13), cardiac valvular EDS (cEDS), and OI/EDS disease
(14,15). Given the clinical and genetic heterozygosity of
these diseases, their diagnosis and treatment are always
challenging, and this is particularly true for rare diseases.
Hence, detailed clinical characteristics of rare diseases
are extremely important. A substitution of c.697-2A>G
in the COL1A1 gene has been reported three times,
though information on clinical phenotypes is limited. The
current report describes a splice site mutation of c.697-
2A>G in COL1A1 that was identified for the first time in
Chinese patients with OI.

2. Case Report

The proband was a 10-year-old girl of normal height
and weight at birth. She was 50 cm tall and weighed 3.3
kg at delivery. She could not walk until she was 1 and
half years old. Delayed closure of the fontanelle was
noted, and pectus carinatum was mild. She suffered a
forearm fracture when she was 11 months old and a
lower leg fracture at the age of 4, as shown on X-rays
(Figure 1). Teeth, hearing, and vision were all normal
in the proband and her father and grandmother, both
of whom had OI. Flat feet, gray-blue sclerae, and pes
planus were present in all three family members. The
proband's affected father and grandmother also had
a relatively normal height and weight, dislocations of
the ankle and elbow, limb muscle weakness, and
upper limb fractures. Joint laxity was not evident in
the proband. The proband had a low bone mass, with a Z
score of -2 in the lumbar spine according to dual energy
X-ray absorptiometry (DXA).

A heterozygous mutation of c.697-2A>G in intron
9 was identified in the proband and her affected family
members (Figure 2) according to molecular analysis (16).

3. Discussion

This report is the first to describe an A to G point
mutation in intron 9 at the -2 position of the splice
receptor site of the COL1A1 gene in a Chinese patient
with OI and her family. There are no Chinese patients
with this mutation according to a literature and database
search (http://oi.gene.le.ac.uk) (8,9). The current patient
and her family members had fractures of the upper
limb, frequent small joint dislocations, blue sclerae,
flat feet, and normal teeth, sight, and hearing. Since an
RNA analysis was not performed, so errors in COL1A1
transcription were not analyzed.

A total of 12 individuals with OI and four different
mutations at position of 697 have been reported;
these mutations include 11 splice site mutations and
1 missense mutation. A splice site mutation of c.697-
2A>G was reported 3 times in patients with OI type
I or IV; one of the three patients with type I OI was
35 years old and presented with blue sclerae, normal
hearing, and bone fractures (17). The mutations -1G>T
and -1G>C at the same site have also been identified in
patients with mild or moderate OI who lacked clinical
symptoms (http://oi.gene.le.ac.uk) (8,9,17). A mutation
of G > C lead to glycine to arginine substitution, which
was identified in one 68-year-old female patient with
type IV OI; the patient did not have blue sclerae or
a history of childhood fractures and extravertebral
fractures, but she had suffered a vertebral fracture once
(18). The splice site of c.697-2delA has been identified
in one 14-year-old Korean female patient with type I OI
(19).

A study has reported that mutations in the 5’
splice donor site and 3’ splice acceptor site consensus

Figure 1. X-rays from the proband. (A) 11 months of age; (B) age 4.

Figure 2. Molecular analysis of a family with OI. (A) Pedigree of the proband’s family; (B) Electropherograms showing the partial sequence of COL1A1 in the proband and her parents.
sequences are related to diseases (7). RNA mis-splicing underlies one of the main types of mutations in patients with OI and type I collagen genes. Type I collagen consists of 2 type I collagen α1 chains and 1 α2 chain coil in a triple helix structure, with 3 repeating amino acids: Gly-X-Y. COL1A1 and COL1A2 contain approximately 52 intronic sequences that are particularly susceptible to RNA splicing mutations (20). A mutation of c.697-2A>G introduces a cryptic splice acceptor site in intron 9 of the COL1A1 gene and may lead to retention of the intron in the abnormally spliced mRNA.

Mutations near the N-telopeptide, which links the N-propeptide to the triple helical domain of collagen, are often associated with aEDS or OI/EDS. Cases of OI/EDS are rarely reported (15, 21-27). The molecular mechanism for OI/EDS is closely related to defective procollagen and cross-linking due to interference with N-propeptide processing (26,28). Patients with OI/EDS have generalized joint hyperlaxity and skin hyperextensibility, early progressive scoliosis, and OI symptoms such as blue sclerae and fractures.

Clinical symptoms of OI and OI/EDS overlap. The relationship between clinical characteristics of those diseases and mutations in the N-terminal region of type I collagen will be more apparent once more data on their clinical phenotypes are available.

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References


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Goldston syndrome with congenital hepatic fibrosis: A rare cause of neonatal cholestasis

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Summary

Goldston syndrome (GS) is a rare association of Dandy-Walker malformation (DWM) and cystic renal dysplasia with or without hepatic fibrosis. It is considered to be a milder variant of Meckel Gruber syndrome (MGS) and shares features with Miranda syndrome. We reported a 22 day old infant with DWM and autosomal recessive polycystic kidney disease (ARPKD) who presented with cholestasis and acholic stools. Ultrasonography and magnetic resonance cholangiopancreatography (MRCP) confirmed the diagnosis of congenital hepatic fibrosis (CHF). The child improved with supportive treatment. CHF is a rare condition which may present as a syndromic association.

Keywords: Dandy walker malformation, cystic renal disease, syndromic association, neonatal cholestasis, fibrocystic disease of liver

1. Introduction

Goldston syndrome (GS) was first described by Goldston in 1963 and is characterized by cystic renal dysplasia with Dandy-Walker malformation (DWM) with or without congenital hepatic fibrosis (CHF) (1). GS has been reported in antenatal cases and among few surviving babies postnatally (2-4). CHF is an autosomal recessive disorder caused by defective development of ductal plate during the embryonal period. It usually presents with features of portal hypertension in older children and adolescents. CHF may be associated with renal or central nervous system malformations as syndromic associations like Joubert syndrome, Bardet Biedl Syndrome and Meckel Gruber syndrome (MGS). GS is a rare cause of CHF.

The following record describes a rare case of a neonate with GS who was diagnosed with CHF with reported survival using conservative management.

2. Case Report

A male baby delivered at term, birth weight 2,750 grams, born of third degree consanguineous marriage presented at 22 days of life with fever, lethargy and jaundice noted for the last three days. Antenatal ultrasound in third trimester detected a large cystic area in posterior fossa communicating with fourth ventricle measuring 3.6 × 4.0 cm with absent cerebellar vermis suggestive of DWM and bilateral enlarged and echogenic kidneys without oligohydramnios. The perinatal course of the baby was uneventful. Postnatal examination did not reveal any dysmorphism, hepatosplenomegaly, external cranial defects, eye abnormality or any cardiac anomaly. Head circumference was normal at birth (35 cm). Ultrasonography on day two of life confirmed the presence of DWM and showed bilateral enlarged (6.3 × 3.1 cm and 6.0 × 3.1 cm) kidneys with increased cortical echogenicity and tiny multiple cysts suggestive of autosomal recessive polycystic kidney disease (ARPKD). There were small cysts in left liver lobe with mild dilatation of intrahepatic biliary radicals. Baby was discharged on day 18 of life on exclusive breastfeeding until he became sick. There was no history of seizures, bleeding manifestations, abdominal distension, edema or decreased urine output. The previous pregnancy was terminated at 18 weeks gestation due to antenatal detection of a central nervous system malformation.

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Autopsy was not done.

At presentation child was lethargic, icteric, febrile with heart rate of 140/min and respiratory rates of 55/min with normal oxygen saturation and weight of 3,100 grams. Head circumference had increased by five cm to 40cm. Blood pressure was normal. There was no pallor or edema. There was firm hepatomegaly (span 8 cm) and splenomegaly (1 cm enlarged). Three consecutive stool samples were observed and found to be acholic. His hemoglobin was 145gm/L, total leucocyte count was 10.1 × 10⁹/L (lymphocytes 65%) and normal platelets. Liver function tests showed total bilirubin of 177.8 µmol/L (direct 76.9 µmol/L), aspartate and alanine aminotransferase as 364 and 789 IU/L respectively. Total protein was 50 g/L with serum albumin of 35 g/L, prothrombin time and INR were normal. Alkaline phosphatase was 902 IU/dL and gammaglutamyl transferase (GGT) was 16.34 ukat/L. The renal functions, serum electrolytes, blood glucose and chest X-ray were normal. Blood and urine cultures were sterile.

A possibility of cholangitis or sepsis induced cholestasis was considered and child was started on intravenous fluids, injection cefotaxime and amikacin and supplements for cholestasis. His fever and general condition improved after 5 days, he became afebrile and started accepting breast feeding. His stools also became pigmented. Ultrasound of the abdomen showed a distended gall bladder with normal post feed contractility, normal bile duct, altered echotexture with increased echogenicity along the portal tracts in liver parenchyma. Bilateral kidneys were enlarged with multiple tiny cysts suggestive of ARPKD. A provisional diagnosis of CHF (with polycystic kidney disease and CNS malformation) was made. Magnetic resonance cholangiopancreatography (MRCP) showed diffusely hypointense liver with linear T2 hyper intense bands along the portal tracts with normal biliary tract suggestive of CHF (Figure 1), diffusely enlarged bilateral kidneys with increased T2 signal intensity and loss of corticomedullary differentiation suggestive of ARPKD (Figure 2). Liver biopsy was not performed. Magnetic resonance imaging (MRI) of brain showed large cyst in posterior fossa, which was communicating with fourth ventricle, with absence of cerebellar vermis suggestive of DWM (Figure 3A and 3B). The syndromic association of CHF, ARPKD and DWM was evaluated and possibility of GS was considered.

Figure 1. Coronal T2 weighted magnetic resonance cholangiopancreatography showing diffusely hypo intense liver parenchyma (asterisk) and periportal T2 hyper intensities (arrows).

Figure 2. Coronal T2 weighted magnetic resonance imaging of abdomen showing enlarged bilateral kidneys with increased T2 signal intensity and loss of corticomedullary differentiation.

Figure 3. Magnetic resonance imaging of the brain. (A), T2 FLAIR sagittal section showing superior displacement of torcular herophili (arrow) with cystic dilatation of fourth ventricle (asterisk); (B), T2 weighted coronal section showing cystic dilatation of fourth ventricle with absence of cerebellar vermis (arrow).
The child was continued on supportive conservative treatment, breastfeeding, and supplements on which his jaundice improved. A cystoperitoneal shunt is planned for DWM.

3. Discussion

GS is a rare syndrome initially detected in antenatal life with constellation of DWM and renal abnormalities (1-3). Moerman et al. reported two surviving siblings with cystic renal dysplasia, central nervous system (CNS) abnormality with cranium bifidum and hepatic ductal plate malformation. One of them had DWM while the other had occipital encephalocele. They proposed that GS was a milder variant of MGS based on similar findings seen in both syndromes (5). Another familial series was reported by Walpole et al. of three siblings with DWM associated with cystic renal dysplasia. CHF was seen in one of these siblings and none of them had polydactyly. They concluded it to be a syndrome distinct from MGS owing to absence of cardinal features of MGS apart from cystic renal dysplasia (6). There are other rare reports of subjects with GS who had survived beyond the perinatal period (4,7). The index case is one of the rare cases surviving beyond neonatal period. Although hepatic fibrosis has been previously reported, there is no reported case of cholestasis in neonate as a manifestation of GS as seen in the index case. Other reported features of GS include facial dysmorphism, hypoplasia of carpus callosum and absence of spleen (4,6). There is familial association involving both male and female suggesting an autosomal recessive (AR) inheritance. Presence of consanguinity and family history in the index case points to AR inheritance. The previous aborted sibling of index case also had brain abnormality presumed to be a similar syndromic association.

The prototype syndrome involving central nervous system and kidneys is MGS. MGS is an autosomal recessive syndrome characterized by cystic renal dysplasia, occipital encephalocele and post axial polydactyly affecting all four limbs (8). All three cardinal features are found in only about half of them (1). Two of these three anomalies are required to make the diagnosis of MGS (9). The incidence ranges from one in 13,000-40,000 (10). A variety of other malformations have been reported in MGS. MGS may be associated with agenesis of cerebellar vermis and posterior fossa cyst suggestive of DWM, cystic hygroma, cardiac malformation, microcephaly, IUGR, and cleft palate (9,11-13). Hepatic fibrosis and bile duct proliferation has also been reported and can be present in up to 1/5th of the cases (4,14). Cystic renal dysplasia is the most constant feature while post axial polydactyly is the most variable feature of MGS (9,11). Most babies are either still birth, medically terminated or die soon after birth (15). The index case did not qualify for MGS due to absence of occipital encephalocele and polydactyly. Another rare syndrome similar to GS is Miranda syndrome, also known as cerebrohepatorenal syndrome, which is characterized by cystic renal dysplasia, DWM and hepatic fibrosis. It is believed that both GS and Miranda syndrome are milder variants of MGS (1). Another syndrome associated with DWM and hepatic fibrosis is COACH syndrome with additional features like ataxia, nystagmus, oculomotor apraxia, developmental delay and facial dysmorphism. Gentile et al reported two siblings with this syndrome, one of them required liver transplantation due to liver failure caused by CHF (16). The index case differs due to presence of renal involvement and absence of other features of COACH syndrome.

CHF is an autosomal recessive fibrocystic disease of the liver. It results from failure of remodeling of the ductal plates during embryonic development leading to persistence of immature duct structures which stimulate hepatic stellate cells to cause progressive periportal fibrosis. Patients commonly present in adolescence or adulthood with features of portal hypertension and cytopenias due to hypersplenism with preserved hepatic synthetic function. In a study on 26 patients with CHF with mean age of 28.4 years, most common symptom was abdominal distension with splenomegaly (43%) followed by recurrent cholangitis (23%) and esophageal variceal bleed (7.7%) (17). Another study involving 25 patients with CHF (mean age 8.5yr) reported hematemesis (60%) and abdominal distension (48%) as common symptoms with 5 (20%) of cases each detected incidentally and on sibling screening (18). Presentation of CHF as neonatal cholestasis is rare (19). The index case presented during neonatal period with features of cholangitis and improved with supportive management. Typical hepatic imaging findings included caudate lobe hypertrophy, periportal T2 hyper intensities and biliary duct anomalies in cases with Caroli syndrome (20). Liver biopsy was not done in the index case but MRCP findings in association with ARPKD were diagnostic of CHF. CHF is associated with many syndromes and common ones being Joubert syndrome, Bardet Biedl syndrome, and MGS (17). Goldston and Miranda syndrome are also associated with CHF.

To conclude, we describe a rare presentation of GS presenting in neonatal period with cholestasis confirmed to be CHF. Determining the syndromic association is important for holistic management and long term prognosis.

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We thank the parents of the patient for allowing us to publish the information about their child that will help increase knowledge about the disease.

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The progress of, challenges faced by, and future of rare disease patient organizations in China

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1. Introduction

In May 2018, five Chinese national agencies jointly published China's First National List of Rare Diseases, which included 121 diseases (1). This is the first time that rare diseases have been clearly delineated by the Chinese Government at the national level (2). Several policies on rare diseases in China have been promulgated since the list was issued. As the government and society pay greater attention to rare diseases, industry has begun to organize a series of charitable activities such as medical aid, public education, and policy advocacy. After nearly 20 years, organizations for Chinese patients with rare diseases have progressed. Many problems still remain, including a relatively small number of organizations, a low level of specialization, a lack of stability, limited social influence, and limited access to social resources. In order to spur the development of Chinese rare disease patient organizations, public education needs to be enhanced, policy support is needed, teams need to be created, and communication and cooperation need to be enhanced.

Summary

In addition to difficulties with treatment and expenses, patients with rare diseases in China greatly lack social support. In around 2000, Chinese patients with rare diseases and their families began to organize a series of charitable activities such as medical aid, public education, and policy advocacy. After nearly 20 years, organizations for Chinese patients with rare diseases have progressed. Many problems still remain, including a relatively small number of organizations, a low level of specialization, a lack of stability, limited social influence, and limited access to social resources. In order to spur the development of Chinese rare disease patient organizations, public education needs to be enhanced, policy support is needed, teams need to be created, and communication and cooperation need to be enhanced.

Keywords: Rare disease, patient organization, China

2. Rare disease patient organizations and their function

A patient organization is a non-profit organization created by patients with a certain disease or family members to represent and advocate for the overall interests of patients, to integrate resources from different stakeholders, and to provide services to the patients or their family (6). Rare disease patient organizations need to be established to provide social support due to the low prevalence of those diseases, the small number of patients, the lack of medical care, the psychological pressure on patients and their families, and the intense sense of loneliness.

Rare disease patient organizations originated in the US since the 1980s and then emerged in Europe and Japan (7). In addition to the functions mentioned earlier, rare disease patient organizations play an increasingly important role in research on rare diseases and orphan drugs, including enrollment in registries, helping to fund research, and participating in clinical trials (8).

3. The development of rare disease patient organizations in China

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The development of rare disease patient organizations in China can be roughly divided into three stages:

*Early Days (2000-2005)* In around 2000, Chinese patients with rare diseases and their families began to assemble and organize a series of charitable activities such as medical aid, public education, and policy advocacy. Chinese patients with hemophilia have taken the lead in establishing a platform for communication and mutual assistance through the Internet, thus opening the way for the development of rare disease patient organization in China.

*On the Rise (2006-2010)* The Chongqing Hemophilia Rehabilitation Association was established in 2006, becoming the first patient organization registered in civil affairs bureau of China (7). In 2008, the China-Dolls Care and Support Association (the predecessor of the China-Dolls Center for Rare Disorders, or CCRD) was created by patients with osteogenesis imperfecta. The first special fund for patients with rare diseases in China, the Dolls Care and Support Fund, was set up by the CCRD under the China Social Welfare Foundation (9).

*Further Development (since 2011)* In 2011, the CCRD held a "Meeting on Capacity-building for Rare Disease Patient Organizations". The meeting focused on how to establish a standardized NGO and the creation of a platform for a rare disease network. Representatives from patient organizations representing 18 rare diseases jointly launched the "China Rare Disease Network." This meeting made many patients with rare diseases aware of the need to establish patient organizations, thereby facilitating the establishment of more rare disease patient organizations.

In 2013, the Chinese Organization for Rare Disorders (CORD), was formally established to create a platform, support the patient community, educate the public, research policy, and cooperate with international counterparts. The CORD has made important contributions to the development of Chinese rare disease patient organizations.

### 4. Challenges faced by rare disease patient organizations in China

Although rare disease patient organizations have developed for several years, there are still quite a number of problems:

*Small number and dispersion of power* There are currently about 80 rare disease patient organizations in China (10), but this number is far from enough compared to the number of rare diseases and patients. Although several preliminary networks of rare disease patient organizations have been created, these organizations remain weak due to different rates of development and a lack of strong cooperation or integration (11).

*Low level of organizational specialization* Most patient organizations have a low level of development and structure. The leader or head of the organization is a patient or family member. Most organizations are not registered. Most have no full-time staff, no fixed office, no consistent source of funding, and no standardized processes or systems.

*Lack of organizational stability* Almost all patient organizations are created and managed by patients and their families. Accordingly, the leader's health will greatly affect the organization's work and development. If a leader is ill or feels pressured by family, financial, or other reasons, the sustainability of the organization's operations will be affected.

*Limited social influence and limited ability to access social resources* Rare disease patient organizations are a new entrant among NGOs in China. Public concern is extremely limited. In addition, the public lacks sufficient understanding of rare diseases. As a result, social resources are neither allocated efficiently nor efficiently, seriously impairing resource allocation.

### 4. Suggestions

*Continued publicity* The urgency of establishing rare disease patient organizations should be publicized. Public education on rare diseases should be continuously provided to attract attention from more groups.

*Policy support* The Government should actively provide more support at all levels to help patient organizations to develop. Patients and patient organizations should be empowered by certain policies, e.g. simplifying the registration process, increasing financial subsidies, and reducing taxes.

*Team building* The current members of patient organizations in China should receive more medical education as well as training in management skills to run those organizations more efficiently and sustainably. The quantity of members should be increased along with their quality. Organizations should recruit professionals with different backgrounds in medicine or NGO management to serve as external committees or consultants.

*Communication and cooperation* Organizations for patients with rare diseases should form alliances and build collaborative networks to the extent possible. This will help them to communicate and cooperate and will help expand educating the public, promoting policies, and securing resources. Patient organizations can also rely on some well-functioning charity organizations to conduct campaigns and secure resources. Patient organizations also need to further cooperate with research institutions and pharmaceutical companies to jointly promote research related to rare diseases. Moreover, communicating with foreign rare disease patient organizations is a way to join relevant collaborative networks and learn from their experience with organizational management.
Acknowledgements

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An up-date on novel molecular targets in testicular germ cell tumors subtypes

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Summary

Testicular germ cell tumors (TGCTs) are the most frequent solid malignant tumors in men 20-34 years of age and the most frequent cause of death from solid tumors in this age group. In addition, the incidence of these tumors has significantly increased over the last few decades. Testicular germ cell tumors are classified into seminoma and nonseminoma germ cell tumors (NSGCTs). NSGCTs can be further divided into embryonal carcinoma, teratoma, yolk sac tumor, and choriocarcinoma. There are noteworthy differences about therapy and prognosis of seminomas and nonseminoma germ cell tumors, even though both share characteristics of the primordial germ cells (PGCs). Many discovered biomarkers including HMGA1, GPR30, Aurora-B, estrogen receptor β, and others have given further advantage to discriminate between histological subgroups and could represent useful molecular therapeutic targets.

Keywords: Testicular germ cells tumors, seminomas, Aurora B, GPR30, PATZ1, HMGA

Testicular germ cell tumors (TGCTs) have the highest incidence among young men (between 20 and 34 years of age) of solid tumors, and their incidence has significantly increased over the last few decades. About 90% of TGCTs are successfully treated with cisplatin-based chemotherapy. However, this kind of therapy raises the possibility of developing secondary cancers and cardiovascular disease. TGCTs are classified into two principal groups: Germ Cell Neoplasias In Situ (GCNIS) that are Seminoma and Nonseminoma (NSE), and spermatocytic tumors that are not GCNIS. NSE tumors encompass embryonal carcinoma, choriocarcinoma, Yolk Sac Tumors (YSTs) and teratomas. TGCTs may develop from a non-invasive type of tumor called carcinoma in situ (CIS): Microscope analysis reveals abnormal cells even though they are still confined inside the membrane of the seminiferous tubules (1-9).

A significant increase in TGCTs incidence occurred in the last few decades, probably due to altered environmental factors that significantly contribute to disease onset. For instance, although the biological mechanisms are still unclear, evidence suggests that the risk of developing TGCTs is associated with maternal smoking during pregnancy, adult height, biomass index, diet rich in cheese, pesticide exposure, and others. Among the risk factors involved in the onset of disease: age, cryptorchidism, family history of testicular cancer, Klinefelter's syndrome, personal history of testicular cancer, congenital abnormalities and infertility. Cryptorchidism is the major risk factor associated with germ cell tumors: it deals with undescended testicle into the scrotum, which remains in the abdomen or groin, thus the risk of developing the disease does not change even after surgery to move the testicle into the scrotum. Remarkably, it is still debatable whether the exposure to some nonsteroidal estrogens during pregnancy, such as diethylstilbestrol (DES) may increase the risk of developing TGCTs. Despite that this divergent evidence confirms the important role played by some environmental factors in TGCTs, etiology has been clearly suggested by migration studies. Consistently, Sweden has an incidence of TGCTs about twice that of Finland and, although first generation migrants from Finland to Sweden show no increased risk, second generation males born to the migrant parents in Sweden present an increased frequency (10,11).
Numerous new biomarkers have been found to discriminate TGCTs subtypes, standing for innovative molecular therapeutic targets. High-mobility group proteins A1 (HMGA1) and A2 (HMGA2) act as powerful diagnostic markers (12-15). Really, these two proteins are diversely expressed in TGCTs in comparison with the stage of tumor differentiation (12,13). For example, HMGA1 binds to other proteins, such as RNF4 (16,17) and PATZ1, which are engaged in transcriptional control and have been demonstrated to be overexpressed are delocalized in human testicular seminomas (18). Currently, we have shown that in human testicular seminomas Estrogen Receptor β (ERβ) expression is strongly down regulated and this down regulation is associated with delocalization of both PATZ1 and HMGA1 transcriptional factors, on the contrary, in normal germ cells, PATZ1 binds to ERβ (19,20).

The serine/threonine kinase NEK2 is a key regulator of centrosome separation and bipolar spindle formation during mitosis and chromatin condensation during meiosis. It controls centrosome separation (essential for the formation of bipolar spindles and high-fidelity chromosome separation) through the phosphorylation of proteins such as CEP250, CROC and NINL, causing their dislocation from the centrosomes. Additionally, NEK2 has a major function in chromatin condensation in the first meiotic division by HMGA2 phosphorylation (21). Moreover, the enhancement and the nuclear localization of NEK2 protein has been found in both seminomas and in seminoma cell line (TCam-2) (22,23). Furthermore, recent studies underlined the new splicing factor kinase function of NEK2 (23).

The RNA-binding protein LIN28 is implicated in the maintenance of the pluripotency of embryonic stem cells, and its expression levels are reduced throughout differentiation. In particular, LIN28 regulates the expression of OCT4 through directly binding to its mRNA transcript in mouse embryonic stem cells. Indeed, LIN28 has a pivotal function for reprogramming somatic cells into pluripotent stem cells. Moreover, LIN28 represents a valid diagnostic marker for testicular GCNIS, classical seminomas, embryonal carcinomas, and YSTs (24). In particular, LIN28 is the main YST marker due to the absence of OCT4 (24).

Estrogen signaling is mediated by two nuclear receptors, estrogen receptor α (ERα) and β (ERβ), that are estrogen dependent transcription factors. ERα is expressed at high levels in human epididymis and efferent ductules, but not in the testis, whereas ERβ is expressed in spermatogonia, spermatoctyes, and in early round spermatids in human testis (1,2). The ERβ subtype is the principal mediator of estrogen action in promoting germ cell survival and development. After activation, these receptors, in association with a myriad of co-activators and repressors, act as nuclear transcription factors for targeted genes. It has been well documented in the literature that ERβ, which is expressed in normal testicular cells, is instead down regulated in seminomas and embryonal cell carcinomas (1,2). Until recently, the estrogen receptors α (ERα) and estrogen receptors β (ERβ) (25-27) have been considered the major physiologic estrogen mediators. Indeed, the G protein-coupled estrogen receptor (GPR30) has proved to have an increasing role in estrogen-mediated signalling in a wide variety of cell types. The critical role of GPR30 in preservation and in development and homeostasis of normal testis is well recognized (28-30). Recent studies show that GPR30 is overexpressed in human spermatogonia, spermatoctyes (29), in the TCam-2 cell line and seminomas. Moreover, it has been verified that ERβ downregulation correlates with GPR30 overexpression both in human CIS and seminomas; furthermore, it has been demonstrated that 17β-estradiol produces ERK1/2 activation through GPR30 (31). Many studies are committed to develop novel therapeutic strategies for the treatment of TGCTs blocking neoplastic germ cells through the design of selective GPR30 inhibitors.

The kinase Aurora-B is another valuable marker able to discriminate among the different tumor histotypes; in fact, it is detected in IGCNU, seminomas and embryonal carcinomas, but not in teratomas and YST. Pharmacological inhibition of Aurora B significantly decreases the cell growth in testicular GC1 and TCam2 cell lines (32-35).

Perturbation of miRNAs plays an important role in the establishment and progression of many cancer types, including TGCTs (36). Although different miRNA signatures are associated with histological subtypes of TGCT, very few miRNAs have been found to have a key role in TGCTs. Indeed, Dicer knockout mice show a premature reduction of germ cell numbers and deregulated differentiation of male germ cells (36). Then, Voorhoeve et al. showed that miR-372 and miR-373 may overcame p53-mediated arrest of the cell cycle (37). Conversely, miR-372 and miR-373 were absent in TGCT-derived cell lines with mutated p53 or expressed low levels of p53, suggesting that these miRNAs may allow the growth of TGCT escaping the p53 checkpoint of the cell-cycle. In this context, data suggests that miR-372 and miR-373 may act as oncogenes in TGCT through the inhibition of LATS2, a tumor suppressor gene (36). Moreover, the novel identification of circulating miRNAs in body fluids like serum, may represent a valid non-invasive manner to diagnosis and follow disease status. In this regard, it has been reported that miR-371 and miR-372 are specifically increased in serum of germ cell tumor patients. Moreover, many other miRNAs have been proposed to be able to discriminate between different tumor histotypes, confirming the function of the embryonic miR-371 and miR-372 in identifying malignant TGCT (38).

Pseudogenes have long been considered as non-
functional genomic sequences. However, recent evidence suggests that many of them might have some form of biological activity, and the possibility of functionality through a microRNA-mediated pathway (39). Recently, two HMGA1 processed pseudogenes (HMGA1P6 and HMGA1P7) were isolated. In particular, these pseudogenes, competing with HMGA1 for microRNA binding, lead to the upregulation of HMGA1 cellular levels, exerting an oncogenic role (40). In this context, although further experiments are needed, preliminary data show that HMGA1 pseudogenes are differentially overexpressed in TGCT histotypes in comparison with normal testis (seminomas, embryonal carcinomas, mixed form teratomas, and YSTs), suggesting a role of HMGA1 pseudogenes in TGCT carcinogenesis.

The development of human TGCTs is subjected to genetic and environmental factors that have a crucial role in deregulating the normal differentiation process in PGCs. Recently, the increasing number of tumor biomarkers has permitted histological discrimination among the various subgroups. A better comprehension of the molecular pathways through which the TGCTs develop will point out new tools to definitely target cancer cells and will help to defeat intrinsic and acquired chemotherapy resistance. Aurora-B serine-threonine kinases, HMGAs and GPR30 inhibitors (41, 42) are promising molecules able to selectively target cancer cells, introducing a new scenario for TGCTs treatment in the near future.

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