ISSN 2186-3644 Online ISSN 2186-361X

IRDR

Intractable & Rare Diseases Research

Volume 12, Number 4
November, 2023

www.irdrjournal.com

IRDR
Intractable & Rare Diseases Research

ISSN: 2186-3644
Online ISSN: 2186-361X
CODEN: IRDRA3
Issues/Year: 4
Language: English
Publisher: IACMHR Co., Ltd.

Intractable & Rare Diseases Research is one of a series of peer-reviewed journals of the International Research and Cooperation Association for Bio & Socio-Sciences Advancement (IRCA-BSSA) Group and is published quarterly by the International Advancement Center for Medicine & Health Research Co., Ltd. (IACMHR Co., Ltd.) and supported by the IRCA-BSSA.

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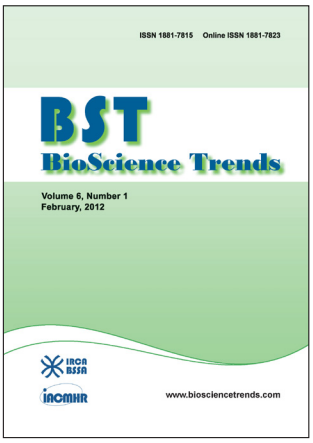

ISSN: 1881-7815
Online ISSN: 1881-7823
CODEN: BTIRCZ
Issues/Year: 6
Language: English
Publisher: IACMHR Co., Ltd.
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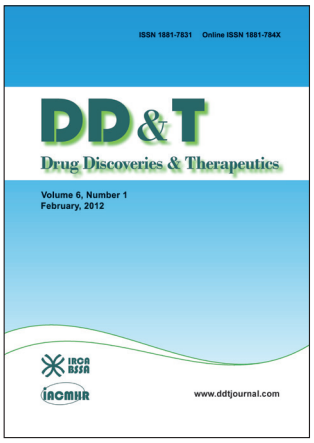
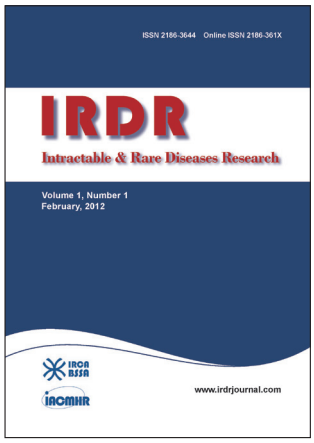


ISSN: 1881-7831
Online ISSN: 1881-784X
CODEN: DDTRBX
Issues/Year: 6
Language: English
Publisher: IACMHR Co., Ltd.
www.ddtjournal.com



ISSN: 2186-3644
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Publisher: IACMHR Co., Ltd.
www.irdrjournal.com

Intractable & Rare Diseases Research

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Tokyo 112-0003, Japan

E-mail: office@irdrjournal.com
URL: www.irdrjournal.com

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DOI: 10.5582/irdr.2023.01091

Artificial intelligence technology in Alzheimer's disease research

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SUMMARY Alzheimer's disease is a neurocognitive disorder and one of the contributing factors to dementia. According to the World Health Organization, this disease has a sig-nificant impact on the global population's health, with the number of affected individuals steadily increasing each year. Amidst rapid technological development, the use of artificial intelligence has significantly expanded into the field of medical diagnostics, encompassing areas such as the analysis of medical images, drug development, design of personalized treatment plans, and disease prediction and treatment. Deep learning, which is an important branch in the field of artificial intelligence, is playing a key role in solving several medical challenges by providing important technical support for the early detection, diagnosis, and treatment of Alzheimer's disease. Given this context, this review aims to explore the differences between conventional methods and artificial intelligence techniques in Alzheimer's disease research. Additionally, it aims to summarize current non-invasive and portable techniques for detection of Alzheimer's disease, offering support and guidance for the future prediction and management of the disease.

Keywords Alzheimer's disease, mild cognitive impairment, artificial intelligence, machine learning, deep learning

1. Introduction

Alzheimer's disease (AD) is a progressive neurological disorder, with the highest in-cidence among individuals ages 65 and older. Current evidence suggests that the age range of the disease is gradually expanding, with middle-aged AD patients under the age of 65 years constituting the younger population affected by AD (*1*). Providing insight into the core mechanisms of AD, Jack *et al*. (*2*) found that the core pathological features of AD are amyloid pathology, tau protein pathology, and neurodegeneration. These three key pathological features are also key to predicting, diagnosing, and treating AD. Before the widespread adoption of artificial intelligence (AI) in healthcare, conventional testing for AD consisted of several approaches. Initially, physicians would rely on their professional expertise to evaluate whether a patient exhibited symptoms of the disease and assess its severity through in-person consultations and inquiries into the patient's medical history. Subsequently, cognitive assessment tools such as the Mini-Mental State Examination (MMSE) and Montreal Cognitive Assessment (MOCA) were used to score the patient's cognitive abilities and determine their cognitive levels (*3*). Now that technology has advanced, magnetic resonance imaging (MRI), positron emission tomography (PET), diffusion tensor imaging (DTI), biomarkers, and cerebrospinal fluid (CSF) (*4*) are gradually being used to detect AD because they do not involve subjective intervening factors. MRI technology uses a strong magnetic field and harmless radio waves to generate high-resolution brain images, aiding physicians in observing the brain structure and detecting potential abnormalities. Biomarkers as described in (*2*) are used to detect AD by labeling specific markers such as amyloid and tau proteins and by ascertaining their presence and the degree of their accumulation. CSF involves the extraction of CSF samples from subjects' spinal region, which are then tested and analyzed to diagnose AD.

However, the aforementioned conventional methods for detection of AD have certain limitations. First, clinical assessment and cognitive testing methods rely to some extent on the subjective judgment of physicians. Second, due to the less distinct pathological features of AD in its early stages, brain imaging techniques such as MRI may lack the sensitivity required to predict this condition (*5*).

2. An overview of AI and its applications

2.1. An overview of AI

AI is a field focused on enabling computer systems to possess cognitive capabilities akin to human thinking. Its objective is to impart machines with the capacity to perceive their surroundings, comprehend natural language, acquire knowledge, engage in logical reasoning, problem-solving, and demonstrate adaptability to varying tasks (*6*). The study of AI extends across diverse domains, predominantly encompassing machine learning (ML) and deep learning (DL).

ML is an important branch in the field of AI that uses algorithms and statistical models to learn from large amounts of data to solve specific tasks (*7*). The core of ML is to perform tasks such as decision-making, classification, and prediction by analyzing and learning the features of data. Common ML models include decision trees (*8*), random decision forests (RFs) (*9*), logistic regression (*10*), support vector machines (SVM) (11), and Bayesian classifiers (*12*).

DL is a subfield of ML. Driven by the proliferation of data and enhanced compu-tational capabilities, ML has evolved into DL (*13*). DL emulates the functioning of neuronal networks in the human brain, acquiring an understanding of data relation-ships through multi-layered neural networks and autonomously extracting data fea-tures. The core of DL is deep neural networks such as the Convolutional Neural Net-work (CNN) (*14*), Long Short-Term Memory Network (LSTM) (*15*), and Transformer (*16*).

2.2. Widespread use of AI in medicine

The applications of AI in medicine are divided into two main categories, physical applications and virtual applications (*17*). Physical applications refer to the use of AI technology to invent and create medical robots and other medical devices in order to assist in medical research and clinical practice. Virtual applications are based on ML and DL and involve algorithmic and software analysis to assist medical research. The scope of virtual applications in healthcare is extensive, including medical testing and treatment, case analysis, and analysis of the progression of chronic diseases. Addi-tionally, the use of virtual applications in the field of AD has garnered significant attention.

The subsequent discussion will focus on the use of AI in the realm of virtual medicine. Its specific applications and potential value in AD detection, diagnosis, and case analysis will be delved into.

3. ML in AD

ML has been used in the field of medical imaging for several decades, with its appli-cations found in computer-aided diagnosis and functional brain imaging (*18*). In the early days, the main task of ML was to assist physicians in identifying and localizing obvious signs of disease. As ML techniques developed and matured, they were gradu-ally used to handle more complex medical detection tasks. By acquiring 18-month longitudinal trajectories of 1,909 patients with mild cognitive impairment (MCI) or AD, an unsupervised ML model, the Conditional Restricted Boltzmann Machine (CRBM), was utilized to simulate the disease trajectories of patients, ultimately doing so in a way that could accurately model the progression of AD (*19*). A study (*20*) summarized multiple brain regions that are closely associated with the pathological mechanisms of AD, including the hippocampus, the internal olfactory cortex, the basal ganglia, the rectus gyrus, the precuneus, and the cerebellum, and it used ML techniques to extract these multivariate biomarkers from structural MRI brain images in order to detect AD early. Studies such as the ones mentioned that utilized ML techniques to detect latent disease markers have made some progress (*20,21,22*). However, ML often requires manual extraction of features, which adds to the difficulty of analyzing large amounts of data.

4. DL in AD

DL performs better and has higher accuracy when dealing with complex data com-pared to conventional ML methods. In addition, DL models are more flexible in of-fering different architectures to adapt to different data characteristics, which is particularly important in AD research.

4.1. Early prediction of AD

The focus of research on early AD is mainly on MCI, because MCI is the transition state between normal aging and AD, and therefore accurate prediction of MCI is im-portant for early prediction of AD (*23,24*). In DL, LSTM performs well in processing time-related data and can be used in prediction problem. Patients' clinical or behav-ioral data usually contain extensive time-series information, and LSTM can capture the features in these time-series and they can be used to predict the changes in pa-tients' cognitive function and disease progression over future time intervals, rather than just a simple categorization of the current patient's disease status (*25,26*). More-over, Hong *et al*. (*25*) focused on predicting AD using five quantitative biomarkers, i.e., the cortical thickness standard deviation (TS), cortical thickness average (TA), WM parcellation volume (SV), surface area (SA), and cortical parcellation volume (CV), and the results of that study showed that all five biomarkers displayed excellent ability to predict AD. TA yielded the best results in prediction. In addition, some studies have explored AD prediction using only retinal pictures, and they have achieved a fairly high accuracy (*27*).

4.2. Diagnosis of AD

DL can be used to build models of disease progression. Unlike early prediction of AD, detection of AD focuses more on patients who already been diagnosed, utilizing DL techniques to assess the extent of cognitive impairment. Nowadays, the diagnosis of AD mostly utilizes MRI images. In the field of processing image data, the CNN ex-cels in processing neuroimaging data, and it can mine important pathologic features from these images to help doctors detect the progression of the patient's disease (*28,29,30*). Other studies have used eye-tracking data to diagnose AD. They track the eye movements and visual focus of test subjects to gather cognitive information (*31*). The studies (*25,26,32*) have integrated early prediction and diagnosis of AD, forming a comprehensive process or framework for predicting AD throughout its course. Medical imaging data are used in pre-trained DL architectures to accurately identify the stage of AD and to help physicians and researchers understand the progression of the condition. In addition, Ho *et al*. (*33*) attempted to use non-invasive near-infrared spectroscopy to diagnose AD, and the highest accuracy (90.91%) was achieved using a CNN-LSTM DL model. These methods enable effective diagnosis of AD in a non-invasive manner and portable format, helping to develop personalized treatment plans and monitor disease progression.

4.3. Treatment of AD

DL plays an important role in the treatment of AD. Transformer is a DL model that can flexibly process different types and lengths of sequence data. In addition, it can capture more detailed feature information. For example, a study used graph neural networks (*34*) to learn and capture structural features of drug molecules, with AD-related ApoE as a target, to search for corresponding acting drugs in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database and PubChem database, to create drug–target interaction (DTI) data, to extract molecular structure information from the DTI data, and to utilize the Transformer network to fuse the features of dif-ferent layers in a graph convolutional neural network to predict potential therapeutics for AD. Amyloid-beta 42 (Aβ-42) is a high-risk factor for triggering AD. In order to predict the efficacy of drugs on AD, Kaushik *et al*. (*35*) used deep neural network technology to screen the PubChem compound library, and they discovered possible Aβ-42 inhibitors and assessed the effects of drugs on AD by observing the effects of inhibitors on Aβ-42.

5. Conclusion

This paper provides an overview of the role of existing AI methods in AD research (Table 1), with a focus

Table 1. Artificial intelligence-based studies related to Alzheimer's disease

Artificial intelligence approach	Main model	Primary data type	Task	*Ref.*
Machine learning	SVM	Magnetic resonance images of brain structures	Diagnosis of AD	*5,18,20*
	Boltzmann machine	Scales, tests, background and other clinical data	Prediction of the whole course of AD	*19*
	SVM, Logistic regression, Decision tree	Longitudinal MRI image	AD prognosis	*21*
	SVM, ANN	CSF data	Diagnosis of AD	*22*
Deep Learning	CNN, LSTM	Multimodal medical imaging, clinical data	AD diagnosis	*4,26*
	VGG-TSwinformer, CNN, LSTM	Magnetic resonance images of brain structures	Detection of early AD	*23,25,28,29,30,32*
	EfficientNet-b2	Retinal image	Detection of AD	*27*
	VGG, ResNet	Eye tracking data	Evaluation of the efficacy of drug therapy in AD	*31*
	CNN-LSTM	Functional near-infrared spectral data	Prediction of AD inhibitors	*33*
	Graph neural networks	Drug and target data	Detection of AD	*34*

on prediction, detection, and treatment. Historically, AD research and diagnosis usually relied on highly specialized techniques and equipment, including CSF, biomarkers, MRI, PET, and DTI. However, the rapid advancement of DL has opened new avenues. Presently, AD can be predicted using eye-tracking data, retinal images, and non-invasive near-infrared technology, offering a more accessible path to early intervention. In addition, DL technology can be used to determine drug efficacy by observing drug-inhibitor interactions, providing a convenient way to personalize treatment. The future will presumably offer more portable and advanced approaches for the prediction, detection, and treatment of AD. DL models are sure to continue playing a pivotal role in this endeavor.

Funding: None.

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

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Received October 6, 2023; Revised October 18, 2023; Accepted October 20, 2023.

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Released online in J-STAGE as advance publication October 23, 2023.

Original Article

DOI: 10.5582/irdr.2023.01068

Challenges associated with delayed definitive diagnosis among Japanese patients with specific intractable diseases: A cross-sectional study

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SUMMARY This study aimed to determine the challenges that cause a delay in the diagnosis of Japanese patients with specific intractable diseases by means of a survey. We conducted a questionnaire survey involving 424 patients with 12 specific intractable diseases. Pearson's chi-square test was used to examine the relationship between diagnostic delay and each factor. The reasons for the diagnostic delay were analyzed. Pearson's chi-square test showed statistically significant differences in the relationship between the period to definitive diagnosis and period between symptom onset and first hospital visit ($p = 0.002$), and the period when the patients suspected the disease ($p < 0.001$). Reasons for diagnostic delay of these patients were patients' time constraints, problem in access to medical institutions, hesitancy in seeking medical attention, and healthcare system issues. Early definitive diagnosis of intractable diseases was hindered by several important issues. The resolution of these issues will require combined societal efforts as well as improvements in the healthcare system. The study revealed the need for improving patients' awareness about their disease, enabling patients to be proactive towards achieving a definitive diagnosis, and making improvements in the healthcare system regarding early diagnosis and care of patients with intractable diseases.

Keywords diagnostic delay, hospital visit, questionnaire

1. Introduction

Rare diseases, as indicated by the term, affect a small number of people; however, there are thousands of rare diseases. Therefore, the number of patients with rare diseases is quite high, and rare diseases are considered critical public health issues. Furthermore, the treatments for 94% of all rare diseases are insufficient, and many patients experience decades of uncertainty and challenges until they reach a definitive diagnosis (*1,2*). The difficulties in diagnosis and access to a specialist also frequently result in misdiagnosis and delayed diagnosis; therefore, it takes a long time for many patients to get a definitive diagnosis (*1,3*).

In addition, the prevalence and general awareness of individual rare diseases are low, resulting in many challenges regarding funds and access to clinical studies that demand international collaborative systems to overcome these barriers (*4*).

Rare diseases are also challenging for physicians to treat. Given the limited information regarding rare diseases, it is not uncommon for primary physicians, who are often the first health care providers with the opportunity to diagnose, to have little experience with rare diseases; hence, referral to a specialist is delayed. Such delays in diagnosis are attributed mainly to physicians lacking experience and information regarding rare diseases (*2*). Therefore, it is necessary to educate physicians about using online and other tools to quickly acquire information on rare diseases (*5*). Early definitive diagnosis is essential for patients with rare diseases because it can help guide them about methods and measures for controlling or slowing disease progression, even if no effective treatment exists (*6*). Furthermore, definitive diagnosis can improve the patient's quality of life (QOL) (*7*).

The European Union (EU) emphasized the importance of supporting "adequate education and training for all health professionals to make them aware of the existence of these diseases and resources available for their care" as actions against rare diseases in 2009 (*8*). The EU has also been the leader in building global data networks for rare diseases (*9*).

The vision of the International Rare Diseases

Research Consortium (IRDiRC) is to "enable all people living with a rare disease to receive an accurate diagnosis, care, and available therapy within 1 year of coming to medical attention" by 2027 (*1*). Japan has been making parallel national and administrative efforts to build medical support networks for intractable diseases (*10*) and shorten the period to definitive diagnosis as much as possible. Such efforts define the "period to definitive diagnosis" as the "undiagnosed period", starting from when the patient first notices physical symptoms to when they seek medical attention. However, the undiagnosed period would be more accurate if it indicates the period from when the patient first notices the symptoms to when they visit a medical institution.

Previous research has identified two challenges affecting the period from symptom identification to the definitive diagnosis of intractable diseases (*11*). The first challenge is related to the high number of patients who wait to make a hospital visit after first noticing physical symptoms, whereas the second is related to the high number of patients from whom it takes a long time to reach a definitive diagnosis, despite the suspicion of the diagnosed disease for a very long time. Determining the reasons for these two challenges will aid in identifying the unmet needs and solutions that will help in shortening the undiagnosed period as well as the period to the definitive diagnosis.

Therefore, the present study addressed two research questions: *i*) why do patients wait to seek medical attention despite noticing physical symptoms, and *ii*) why does it take a long time for patients to reach a definitive diagnosis, despite suspecting the diagnosed disease for a very long time? This study aimed to address these research questions from the patient's perspective and identify the unmet needs.

2. Materials and Methods

We conducted an online questionnaire survey that included 488 participants with 12 specific intractable diseases recruited from a patient panel owned by Rakuten Insight, Inc., in February 2023. After excluding patients whose data could not be analyzed, 424 patients were finally included in the analysis. The data collection process in this study is shown in a flow chart (Figure 1). Furthermore, the various specific intractable diseases and the number of patients with each disease are presented in Table 1.

The questionnaire items consisted of basic sociodemographic data (age, sex, and location of residence), misdiagnosis experience, number of hospital visits until reaching a definitive diagnosis, time from the first medical consultation to reaching the current diagnosis, and period between first noticing the physical symptoms and the first medical consultation. These responses were selected from a list of options. The data from 424 patients were analyzed statistically using the following methods. A subanalysis of 133 patients – who took ≥ 6 months between first noticing physical symptoms and their first medical consultation – was performed to determine the reasons for delay in visiting a medical institution despite experiencing physical symptoms. Another subanalysis of 66 patients who suspected the diagnosed disease for ≥ 6 months was performed to explore the reasons why making a definitive diagnosis took a long time despite suspicions of the current diagnosis, which they answered by selecting responses from among several options. The questionnaire included a space for writing free responses to enable those who selected the "other" option to provide a specific reason. In addition, 141 patients, for whom it took ≥ 1 year to reach a definitive diagnosis, answered the following two questions: "What actions do you think you could have taken to shorten the period to a definitive diagnosis?" and "What kind of environmental or systemic changes do you think could have further shortened the period to definitive diagnosis?"

The IRDiRC's vision is to "Enable all people living with a rare disease to receive an accurate diagnosis, care, and available therapy within 1 year of coming to medical attention by 2027" (*1*). However, due to the lack of a standard definition for "delay to diagnosis", the

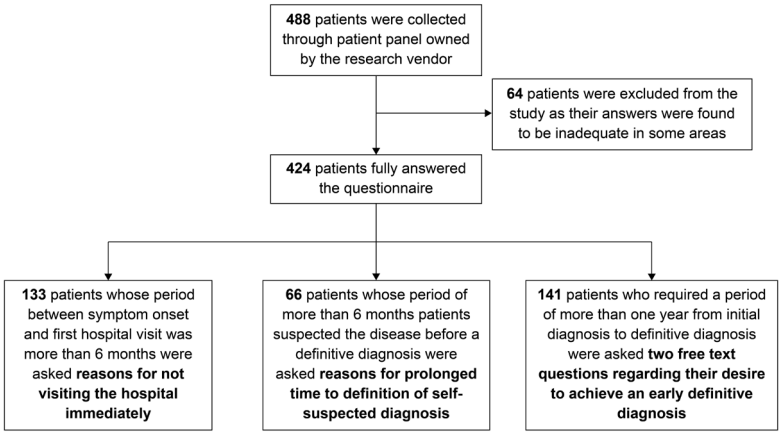

Figure 1. Flow chart of data collection process.

consortium guideline was used as the standard, defining diagnostic delay as ≥ 1 year from the first medical consultation to a definitive diagnosis.

2.1. Sample size

Sample size calculation was based on the following criteria: 5% margin of error, 95% confidence level, and 50% expected response rate. The sample size required for this study was at least 384 cases. The number of patients for each disease designated as intractable was used as a reference for the health administration reports published by the Ministry of Health, Labor and Welfare (*12*).

2.2. Analysis

IBM SPSS, ver28, (IBM Corp. Released 2021. IBM SPSS Statistics for Windows, Version 28.0. Armonk, NY: IBM Corp) was used for statistical analysis.

The eligible data of 424 patients were included in the statistical analysis. Correlations between the period to definitive diagnosis (Diagnosis in 1 year and Diagnosis delay) and misdiagnosis experience, number of hospital visits, period between symptom onset and first hospital visit, and duration for which patients suspected the disease were analyzed using Pearson's chi-square test. Statistical significance was set at $p < 0.05$.

Thematic analysis was used for qualitative data (*13*). The analysis was performed using MAXQDA software 2022 (VERBI Software, 2021). We categorized the code into themes and clustered the themes. Themes, subthemes, and quotes are displayed in tabular form to facilitate the understanding of the phenomenon described.

The reason for not consulting a medical institution immediately, despite noticing physical symptoms, was selected by the respondents from among 12 options (multiple selections were allowed). Those who selected "other reasons" could write the specific reason in the free response form. The responses were interpreted and classified into the following themes: A) Factors associated with patients' access to healthcare facilities, B) time constraints for patients, C) patient-sided psychological factors, and D) other factors. Regarding the reason why it took a long period to reach a definitive diagnosis despite suspecting the diagnosed disease, patients were required to answer by selecting one or more from among seven options. Those who selected "other reasons" could also write the specific reason in the free response form. These options were classified into the following themes: A) Problems regarding medical facilities and healthcare systems, B) factors associated with one's actions, C) factors associated with situational changes, and D) other reasons.

2.3. Ethical approval

The data used in this study were consigned to Rakuten Insight, Inc. and obtained using the company's panel. All data obtained were fully anonymized before analysis. We had no access to the correspondence tables of anonymization or other information that could be used to identify the individuals. Therefore, this study used a completely anonymized questionnaire survey and was carried out in accordance with the method designated by the Research Ethics Review Committee, School of Health Innovation, Kanagawa University of Human Services. The same committee indicated that ethics approval was not required. The study notification number was SHI No. 59. The study's aims were explained to all the participants, who provided informed consent before participating in the survey.

3. Results

As shown in Table 1, this study focused on 12 diseases designated as specific intractable diseases in Japan, and analyzed the data of 424 patients.

The participant characteristics are shown in Table 2. Patients in their 60s accounted for the largest proportion of participants (30.7%), followed those aged 29–49 years (29.2%), 50–59 years (25.5%), > 69 years (13.0%), and < 29 years (1.7%). Furthermore, 27.1% of the patients experienced misdiagnosis. Notably, 8.5% of the patients took 6–12 months between first noticing physical symptoms and seeking medical attention for the first time, whereas 22.9% waited for > 1 year. Furthermore, 2.1% of the patients suspected having the diagnosed disease for 6–12 months, whereas 13.4% suspected having it for > 1 year. Notably, for 33.3% patients, the time from the first medical consultation to the definitive diagnosis was ≥ 1 year.

Next, Pearson's chi-square test was used to examine the correlations between the period to definitive diagnosis (diagnosis in 1 year and diagnosis delay) and misdiagnosis experience, number of hospital visits, period between symptom onset and first hospital visit, and duration for which the patients suspected the disease (Table 3). Chi-square test showed a statistically significant relationship between misdiagnosis and the period to definitive diagnosis ($p = 0.042$) (Table 3a). A relationship was also observed between misdiagnosis and delayed diagnosis. The correlation between the number of hospital visits until reaching a definitive diagnosis and the period to definitive diagnosis is shown in Table 3b, and a significant correlation was observed between both items ($p < 0.001$). A relationship was also observed between the number of hospital visits and the period to definitive diagnosis. There was a significant difference between the period to definitive diagnosis and the period between symptom onset and first hospital visit ($p = 0.002$) (Table 3c). Delayed definitive diagnosis occurred in 44.4% of patients who took 6–12 months between symptom onset and their first hospital visit, and in 44.3% of those who took > 1 year, clearly indicating that the

Table 1. Patients with specified intractable diseases registered in this survey

Specified intractable disease	Number	Percentage (%)
Crohn's disease	46	10.8
Sjögren's syndrome	45	10.6
Polycystic kidney disease	47	11.1
IgA nephropathy	50	11.8
Systemic lupus erythematosus	48	11.3
Parkinson's disease	30	7.1
Idiopathic dilated cardiomyopathy	28	6.6
Multiple sclerosis/neuromyelitis optica	28	6.6
Spinocerebellar degeneration (excluding multiple system atrophy)	19	4.5
Idiopathic interstitial pneumonia	40	9.4
Eosinophilic sinusitis	29	6.8
Spinal muscular atrophy	14	3.3

rate of delayed definitive diagnosis increased when the patients waited for > 6 months to seek medical help after the onset of symptoms. Furthermore, the patients who waited for a long time between first noticing physical symptoms and visiting a medical institution also experienced a long duration between the first medical consultation and definitive diagnosis. Furthermore, a significant difference was found between the period when patients suspected the disease and the period to definitive diagnosis ($p < 0.001$), as shown in Table 3d.

Furthermore, 55.6% of the patients who suspected the disease for 6–12 months and 66.7% who suspected the disease for > 1 year experienced delayed definitive diagnosis, indicating that patients who suspected the disease for ≥ 6 months experienced delayed definitive diagnosis. To explain the reason for these findings from the patient's perspective, we asked the patients who took ≥ 6 months to visit a medical institution despite noticing physical symptoms why they avoided visiting a medical institution, and their responses are presented in Table 4. The patients selected their answers from 12 options, and those who selected "other", described the specific reason in a free-response form. The most frequently cited reason was that they "decided to wait and see" (36.2%), followed by they "did not know which medical institution to visit" (15.1%), they thought "it was inconvenient to go to a medical institution" (9.3%), they "did not have time to visit a medical institution" (7.8%), they faced "difficulty making an appointment" (4.1%), they were "afraid of receiving a diagnosis" (4.1%), they were "afraid that a definitive diagnosis would affect their work or education" (3.8%), they had "no nearby medical institution" (3.5%), they were "afraid to visit a medical institution" (3.2%), and they had "financial concerns that prevented them from seeking medical help" (2.6%). In addition, 2.6% of the patients "did not consider it at all", and 7.8% provided "other reasons". These responses were interpreted and classified into four themes as follows: A) factors associated with patients' access to healthcare facilities, B) time constraints for patients, C) patient-sided psychological factors, and D) other factors, accounting for 32.0%, 44.0%, 11.1%, and 13.0% of the patients, respectively, as shown in Figure 2. Psychological factors seem to stem from problems such as the inability to continue working because the name of one's disease is revealed by visiting a medical institution, or hesitation to visit a medical institution because of prejudice from others.

Table 2. Characteristics of the study sample

Characteristic	Number	Percentage (%)
Sex		
Male	256	60.4
Female	168	39.6
Age		
< 29 years	7	1.7
29–49 years	124	29.2
50–59 years	108	25.5
60–69 years	130	30.7
> 69 years	55	13.0
Misdiagnosis experience		
Yes	115	27.1
No	309	72.9
The period between symptom onset and first hospital visit		
Immediately after symptom onset	76	17.9
< 1 month	130	30.7
1–6 months	85	20.0
6 months–1 year	36	8.5
> 1 year	97	22.9
The duration for which patients suspected the disease before a definitive diagnosis		
Never doubted	251	59.2
< 1 months	74	17.5
1–6 months	33	7.8
6 months–1 year	9	2.1
> 1 year	57	13.4
The period from first hospital visit to definitive diagnosis		
Diagnosis in 1 year	283	66.7
Diagnosis delay	141	33.3

Table 5 presents the answers to the question regarding the reasons for the prolonged time to definitive diagnosis, despite having suspected the disease for ≥ 6 months. The most frequently cited reason was "extensive tests were conducted before the definitive diagnosis of the current medical condition, but the cause remained elusive, leading to a prolonged duration" (33.3%), followed by "prior misdiagnosis at previous healthcare facilities led to a belief in the incorrect diagnosis and subsequent delay in seeking specialized medical care" (17.2%), "lack of recommendation from physicians to seek specialized medical care contributed to the delay in visiting a specialized healthcare institution" (15.1%), "being busy and postponing their own visit to a specialized medical facility" (12.9%), and "limited access to specialized healthcare institutions in the residential area posed difficulties in seeking medical consultation" (8.6%). Furthermore, 2.2% of the patients responded that "the recent COVID-19 situation prevented proactive visits to hospitals for medical consultation" and 10.8% cited "other reasons". As shown in Figure 3, the themes A) problems regarding medical facilities and healthcare systems, B) factors associated with one's actions, C) factors associated with situational changes, and D) other reasons, accounted for 74.2%, 12.9%, 2.2% and 10.8%

Table 3. Distribution of each independent variable and the definitive diagnosis period

	Study sample *n* (%)	Diagnosis in 1 year *n* (%)	Diagnosis delay *n* (%)	*p*	χ^2 for trend
3a. Distribution of misdiagnosis experience and the definitive diagnosis period					
Misdiagnosis experience					
Total	424 (100)	283 (66.7)	141 (33.3)	0.042	4.122
Yes	115	68 (59.1)	47 (40.9)		
No	309	215 (69.6)	94 (30.4)		
3b. Distribution of the number of hospitals visited in the period before and after a definite diagnosis was made					
Number of hospital visits					
Total	424 (100)	283 (66.7)	141 (33.3)	< 0.001	22.569
1 visit	156	114 (73.1)	42 (26.9)		
2 visits	164	118 (72.0)	46 (28.0)		
3 visits	63	35 (55.6)	28 (44.4)		
> 3 visits	41	16 (39.0)	25 (61.0)		
3c. Distribution of the period between symptom onset and first hospital visit and the definitive diagnosis period					
Period between symptom onset and first hospital visit					
Total	424 (100)	283 (66.7)	141 (33.3)	0.002	16.864
Immediately after symptom onset	76	46 (60.5)	30 (39.5)		
< 1 month	130	100 (76.9)	30 (23.1)		
1–6 months	85	63 (74.1)	22 (25.9)		
6 months–1 year	36	20 (55.6)	16 (44.4)		
> 1 year	97	54 (55.7)	43 (44.3)		
3d. Distribution of the period between suspicion of the disease and definitive diagnosis					
The duration for which patients suspected the disease before a definitive diagnosis					
Total	424 (100)	283 (66.7)	141 (33.3)	< 0.001	36.847
Never doubted	251	184 (73.3)	67 (26.7)		
< 1 month	74	54 (73.0)	20 (27.0)		
1–6 months	33	22 (66.7)	11 (33.3)		
6 months–1 year	9	4 (44.4)	5 (55.6)		
> 1 year	57	19 (33.3)	38 (66.7)		

Table 4. Reasons for delayed medical consultation despite perceiving physical symptoms

Option number	Description	Quantity of responses (%)	Clustered theme
1	I decided to wait and see.	125 (36.2)	B
2	I did not know which medical institution to visit.	52 (15.1)	A
3	It was inconvenient to go to a medical institution.	32 (9.3)	A
4	I didn't have time to visit a medical institution.	27 (7.8)	B
5	Lack of convenience in the medical institution (*e.g.*, difficulty in making appointments).	14 (4.1)	A
6	I was afraid of receiving a diagnosis.	14 (4.1)	C
7	I was afraid that a confirmed diagnosis would affect my work or education.	13 (3.8)	C
8	There was no nearby medical institution.	12 (3.5)	A
9	I was afraid to visit a medical institution.	11 (3.2)	C
10	Financial concerns prevented me from seeking medical help.	9 (2.6)	D
11	I didn't consider it at all.	9 (2.6)	D
12	Other reasons.	27 (7.8)	D

of responses, respectively. These results suggested that the main reasons for delayed definitive diagnosis despite suspecting the disease for ≥ 6 months were associated with problems regarding medical facilities and healthcare systems.

Next, patients who took > 1 year to reach a definitive diagnosis were asked the following questions: "What actions do you think you could have taken to shorten the period to a definitive diagnosis?" and "What kind of environmental or systemic changes do you think could have further shortened the period to definitive diagnosis?" Notably, 141 patients who took > 1 year to reach the definitive diagnosis provided free-response answers that were qualitatively analyzed and classified

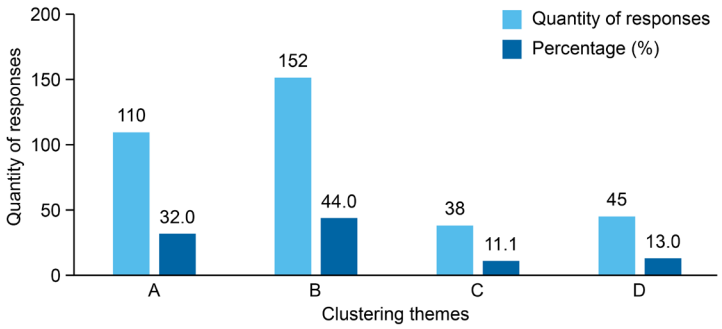

Figure 2. Clustering themes of reasons for delayed medical consultation despite perceiving physical abnormalities. (A) Factors related to patients' access to healthcare facilities; **(B)** Factors related to the time challenge of getting to the hospital; **(C)** Psychological factors; **(D)** Other factors.

Table 5. Reasons for prolonged time to the definition of self-suspected diagnosis

Option Number	Description	Quantity of responses (%)	Clustered theme
1	Extensive tests were conducted before the definitive diagnosis with the current medical condition, but the cause remained elusive, leading to a prolonged duration.	31 (33.3)	A
2	Prior misdiagnosis at previous healthcare facilities led to a belief in the incorrect diagnosis and subsequent delay in seeking specialized medical care.	16 (17.2)	A
3	Lack of recommendation from physicians to seek specialized medical care contributed to the delay in visiting a specialized healthcare institution.	14 (15.1)	A
4	Because I was busy and postponed my own visit to a specialized medical facility.	12 (12.9)	B
5	Limited access to specialized healthcare institutions in the residential area posed difficulties in seeking medical consultation.	8 (8.6)	A
6	The recent COVID-19 situation prevented proactive visits to hospitals for medical consultation.	2 (2.2)	C
7	Other reasons.	10 (10.8)	D

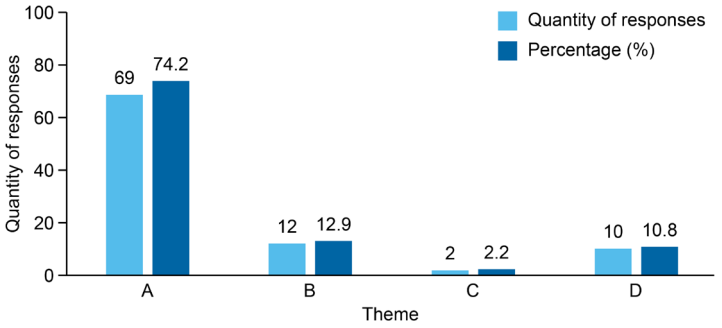

Figure 3. Clustering themes of reasons for prolonged time to the definition of self-suspected diagnosis. (A) Factors related to the medical facility or health care system; **(B)** Factors related to a decrease in one's willingness to visit a doctor; **(C)** Factors related to changes in circumstances; **(D)** Other factors.

into several themes and subthemes.

The free-response answers to the first question are presented in Table 6. The most frequently cited response was "having a strong interest in one's own symptoms" (n = 75), followed by "cannot be improved by one's own actions alone" (n = 48). Regarding "having a strong interest in one's own symptoms", the patients answered that having an interest in their symptoms would have led to them actively seeking medical attention, researching for information about their disease, and even seeking a second opinion. Regarding "cannot be improved by one's own actions alone", the patients expressed that attempts to change their behaviors would not have accelerated the process of reaching a diagnosis; however, improved efficiency of the healthcare system and access to health screening data might have shortened their time to diagnosis. Relationship of trust with the physician was another factor that patients indicated as a condition for effectively reaching an early diagnosis. In addition, topics related to the COVID-19 pandemic were also mentioned.

Next, the free responses to the second question are presented in Table 7. The most frequently cited response was "better systems for early diagnosis" (n = 105), where the respondents mentioned the importance of correcting regional disparities in terms of effective use of health

Table 6. Themes from the free text comments regarding what actions the patients themselves could have taken to shorten the time to a definitive diagnosis

Theme	Subthemes	Number	Quotes
Having a strong interest in one's own symptoms	Arranging work schedules to decide on early medical consultation	75	*I was unable due to my work engagements. (Respondent 187)* *I didn't go to a hospital even though my symptoms were getting worse. I wonder whether my condition would have changed if I had visited a hospital earlier, so I believe it's necessary to take early action. (Respondent 70)*
	Active use of second opinion doctors		*I should've taken advantage of a second opinion more actively. (Respondent 404)*
	Independently researching on the disease		*I should've been more interested in my own body and told someone about it, even about small things like symptoms. (Respondent 304)* *I should have contacted the appropriate institution to get access to a medical consultation that would have allowed me to find the appropriate medical facility. (Respondent 332)*
Cannot be improved by one's own actions alone	Building a healthcare system that allows early diagnosis	48	*It would accelerate the process if we could go directly to a specialized institution, such as a national hospital, without a referral. (Respondent 155)*
	Effective and active use of health screening data		*There was a long watch-and-wait period after getting the test results. While waiting for the next health checkup, I should have visited other medical institutions, or been re-tested. (Respondent 268)* *There was nothing I could do about it. It's a problem of cooperation between medical institutions (or lack thereof). (Respondent 38)*
Environmental changes	Effects of the COVID-19 pandemic	1	*At first, I consulted my family doctor and had the family doctor write a referral to a university hospital. However, it was summer, and I didn't have the energy to put a mask on and go out, and it would have taken a long time to walk, so I could not go out.* *It was very tough walking, dragging my feet. (Respondent 101)*
Building a relationship of trust with the physician	Building a relationship of trust with the physician	3	*I should have built a relationship of trust with the physician. (Respondent 298)*
I do not know		14	

Table 7. Themes from free text comments regarding requests for improvements in the medical environment and system

Theme	Subthemes	Number	Quotes
Better systems for early diagnosis	Effective use of health screening data	105	*would be referred to a doctor with knowledge and experience, not just someone who reads the health screening results. (Respondent 268)*
	Support to take advantage of a second opinion		*It's nice that we have this system to seek a second opinion, but how useful is it really?? When you don't know what disease you have, it is hard to find a new hospital, so active referral by the physician would be helpful. (Respondent 482)*
	Definitive diagnosis by the family physician		*The system should have the family physician make a definitive diagnosis before they refer the patient to a specialized hospital (in my case, a university hospital). That is, it would be great if the patient can find out that they have a specific intractable disease as early as possible. (Respondent 69)*
	Increased accuracy of tests		*I thought it would accelerate the process to definitive diagnosis if all the tests could be done at once. (Respondent 356)* *It would be ideal if we weren't sent to specialized departments, but to a physician or department who can make a multifaceted, overall assessment so that we are not left with the diagnosis "reason unknown". (Respondent 39)*
	Medical fee coverage support		*My disease is not severe, so I would not get financial assistance even if my diagnosis is confirmed. The tests that led to my definitive diagnosis did cost a lot of money, though. I'm sure it depends on the disease, but financial support to get the diagnosis may increase the number of people who get the definitive diagnosis.(Respondent 148)*
	Accessibility of hospital consultations		*It is so hard to get an appointment. I get completely exhausted while waiting at the hospital. It might have made a difference somewhat if these problems were solved. (Respondent 408)* *hospitals opening on Saturdays and Sundays. (Respondent 37)*
	Correcting regional disparities in medical service accessibility		*I think it's a problem that there are no specialists in rural areas. (Respondent 77)*
Having a strong interest in one's own symptoms	Strong motive to come to terms with one's illness	3	*It was my own problem, so it's not so much a matter of the environment or systems, but a problem of individual mentalities. I mean, most people won't imagine getting such a disease. (Respondent 70)*
Building a relationship of trust with the physician	Building a relationship of trust with the physician	1	*relationship of trust with the physician. (Respondent 298)*
I do not know		32	

screening data, supporting second opinions, definitive diagnosis by the family physician, increased accuracy of tests, medical fee coverage, and accessibility to hospital visits.

Regarding "Having a strong interest in one's own symptoms", the respondents indicated the importance of having a strong motive to come to terms with one's illness. The theme of "building a relationship of trust with the physician" also emerged.

4. Discussion

As shown in Table 3c, many patients who waited a long time between the first onset of symptoms and the first medical consultation took a long time to reach a definitive diagnosis, even after seeking medical attention. To determine the reasons for this phenomenon, we surveyed the reasons why the patients did not immediately visit a hospital (Figure 2); the reasons were mainly associated with time constraints for patients and access to medical institutions in most cases (76%). These patients seemed to either not think too deeply that they might be affected by an intractable disease or were aware that it was possible but did not think too much about it. Therefore, boosting incentives for these patients to seek medical attention, such as providing information about diseases to patients, would increase their chances of getting an early diagnosis. Regarding patients' psychological factors, which accounted for 11.1% of participants, it seemed unlikely that mere attempts to boost their motivation would lead to an early diagnosis because they experienced anxiety about visiting medical institutions. Many patients who hesitate to seek medical care are concerned about the potential consequences of a definitive diagnosis, such as becoming unable to continue their current employment, losing their source of income, or facing prejudice from their workplace or the communities they are involved in. Therefore, we need to not only to address medical issues but also foster a broader societal transformation that embraces patients with intractable diseases and makes them feel included in society.

Regarding the reasons it took a long time to reach a definitive diagnosis despite the patient suspecting the disease for > 6 months, problems regarding medical facilities and healthcare systems were the most frequent reasons (Figure 3). Patients seemed to have a strong desire for improvements in the healthcare system. Furthermore, when the patients who took > 1 year to reach a definitive diagnosis were asked, "what actions do you think you could have taken to shorten the period to definitive diagnosis?", the most frequently cited reason was "having a strong interest in one's own symptoms". Patients' research about the disease and actively seeking a second opinion were also positive actions to avoid delayed definitive diagnosis. Notably, patients mentioned that the presence of a physician they trusted might shorten the time to definitive diagnosis and time to the introduction of appropriate care; however, this was limited to a minority. Our previous research showed that patients who experienced delayed diagnosis scored low on parameters of trust in their physician (*11*). This topic needs further investigation.

Our results suggested the importance of changes to medical facilities and systems and ways to ensure the patients are motivated to seek medical attention to shorten the period to the definitive diagnosis of intractable diseases. In such cases, motivation should not only involve encouraging them to visit a medical institution but also involve creating a social environment that is accepting of patients with intractable diseases and where patients and their families can obtain the latest information.

Researchers studying rare diseases are also obtaining a broad spectrum of precise associated data. Patients with rare diseases are geographically dispersed, and it is difficult to aggregate information into a single database. However, recent efforts are made using social media platforms to help find patients with similar intractable health problems and also clinicians with expertise in rare diseases. It is aimed to promote sharing of information on symptoms, treatments, side effects, other diseases and activities, and other various data types beyond those typically captured in a clinical setting or patient registry (*14*). In addition, Klein *et al.* (*15*) recently used Twitter to receive the data on rare health-related problems reported by patients and found it useful for collecting patient-centered information that can be used in future epidemiological analyses.

Yamaguchi *et al*. (*16*) are exploring how data from the medical histories of patients with rare diseases posted on social media can capture patients' perspectives on their health status and assist in speeding up the timeline to diagnosis and treatment. Such an initiative would be useful to researchers and also could motivate undiagnosed patients worldwide to seek medical attention.

The present study identified two important aspects: Firstly, patients who spend a long time until their initial consultation tend to experience a prolonged duration from the initial consultation to a definitive diagnosis, and we have identified the reasons behind this phenomenon. Secondly, there is a prevalent trend among patients who have harbored suspicions about their own illness for an extended period but still experience delays in receiving a definitive diagnosis. We identified the factors contributing to this problem.

However, this study also has some limitations. First, the data was collected through a questionnaire survey aimed at collecting patient input from their perspective. To gain further in-depth patient insight, conducting a survey through interviews is also necessary. In particular, interviews could have provided deeper insights regarding the reasons why the patients were reluctant to visit

healthcare facilities. However, we aimed to obtain the largest possible sample size and broad range of information. Therefore, we believe that the questionnaire-based approach used in our study was appropriate for this purpose. In future, an in-depth investigation regarding the reasons for combining the interviews with surveys may become necessary. Furthermore, future studies should also conduct surveys of physicians to gain a better understanding of challenges experienced in healthcare facilities and identify other unmet needs that lead to delayed diagnosis of patients with intractable diseases.

This study has raised several important issues regarding early definitive diagnosis of intractable diseases. Initiatives by organizations such as IRDiRC and governmental organizations aim to shorten the duration between when patients visit a medical institution and when they receive a definitive diagnosis. However, we believe that the term "undiagnosed period" should refer to the duration from when patients first notice bodily changes to the point at which they receive a definitive diagnosis. In this study, it was observed that many patients who did not immediately seek medical help upon noticing bodily changes and those who spent a long duration until seeking care also experienced diagnostic delay. Moreover, patients who harbored suspicions about their own illness for an extended period also experienced diagnostic delay. In this study, we have elucidated the underlying factors behind why these patients experience delays in receiving a definitive diagnosis.

To fundamentally address these issues, it is crucial to not only focus on improving the current healthcare system, which the government is currently undertaking, but also to raise awareness among patients about their conditions, promote proactive efforts toward obtaining a definitive diagnosis, and foster a societal transformation that embraces patient with intractable diseases and addresses these medical challenges.

Funding: None.

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

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Received August 18, 2023; Revised September 18, 2023; Accepted October 15, 2023.

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Released online in J-STAGE as advance publication October 23, 2023.

Original Article

DOI: 10.5582/irdr.2023.01076

Circ_KIAA0922 regulates Saos-2 cell proliferation and osteogenic differentiation by regulating the miR-148a-3p/SMAD5 axis and activating the TGF-β signaling pathway

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SUMMARY Circular RNAs (circRNAs) are emerging as important regulators in human disease, but their function in osteoporosis (OP) is not sufficiently known. The aim of this study was to identify the possible molecular mechanism of circ_KIAA0922 in osteogenic differentiation of Saos-2 cells *in vitro* and the interactions among circ_KIAA0922, miR-148a-3p, and SMAD family member 5 (SMAD5). Circ_KIAA0922, miR-148a-3p, and SMAD5 were overexpressed by transient transfection. Dual-luciferase reporter assay system was used to analyze the combination among circ_KIAA0922, miR-148a-3p, and SMAD5. In addition, the levels of circ_KIAA0922, miR-148a-3p, SMAD5, osteocalcin (OCN), and runt-related transcription factor 2 (RUNX2) were detected using RT-qPCR or western blot analysis. Alizarin red staining was performed to analyze the degree of osteogenic differentiation under the control of circ_KIAA0922, miR-148a-3p, and SMAD5. We found that circ_KIAA0922 knockdown inhibited the proliferation and osteogenic differentiation of Saos-2 cells. Circ_KIAA0922 directly targeted miR-148a-3p, and miR-148a-3p inhibition reversed the effects of circ_KIAA0922 knockdown on the proliferation and osteogenic differentiation of Saos-2 cells. Overexpression of SMAD5 promoted the proliferation and osteogenic differentiation of Saos-2 cells and attenuated the inhibitory effect of miR-148a-3p on cell proliferation and osteogenic differentiation. In conclusion, circ_KIAA0922 facilitated Saos-2 cell proliferation and osteogenic differentiation *via* the circ_KIAA0922/miR-148a-3p/ SMAD5 axes *in vitro*, thus providing insights into the mechanism of osteogenic differentiation by circ_ KIAA0922.

Keywords Circ_KIAA0922, miR-148a-3p, SMAD5, cell proliferation, osteogenic differentiation

1. Introduction

Bone homeostasis is a dynamic balance between bone formation by osteoblasts and bone resorption by osteoclasts (*1*). In case of an imbalance, various destructive bone diseases such as OP occur (*2*). OP is a debilitating bone disease with a high prevalence worldwide and is the most common in older individuals, especially in postmenopausal women (*3*). Because the incidence of osteoporotic fractures increases with advancing age, measures to diagnose and prevent OP and its complications are of major public health importance (*4,5*). There is accumulating evidence that epigenetic modifications may be mechanisms underlying the links of genetic and environmental factors with increased risk of OP and bone fracture. It has been shown that some forms of RNAs, such as microRNAs (miRNAs), long noncoding RNAs (lncRNAs), and circular RNAs (circRNAs), are epigenetic regulators with significant involvement in the control of gene expression, and as such they affect multiple biological processes, including bone metabolism.

CircRNAs are a new type of noncoding RNAs with a closed continuous loop structure formed by covalent concatenating of the 3'-poly(A) tails and 5'-end capping splice sites. They were first found in viroid in 1976, but are widely distributed in nature (*6*). Previous studies have demonstrated that some circRNAs have a function of microRNA "sponges", which can offset microRNA-mediated mRNA inhibition (*7*), thereby participating in epigenetic regulation and transcriptional and posttranscriptional regulation by different mechanisms

(*8*). There is substantial evidence that circRNAs have a major role in the development of OP, and their great potential as biomarkers and therapeutic targets for OP is being elucidated (*9,10*). It has been shown that circRNAs are differentially expressed in postmenopausal OP. Target prediction and dual luciferase reporter gene assay have shown that circ_0016624 sponge inhibits miR-98 and circ_0048211 sponge inhibits miRNA-93-5p, which increases BMP-2 expression and promotes the transformation of stem cells into osteoblasts (*11,12*). According to Yao *et al*., senile OP is associated with the regulation of circRNA expression (*13,14*). Next-generation sequencing and RT-qPCR have revealed that circ_KIAA0922 is downregulated in a murine asthma model group compared with the control group (*15*). Xie *et al*. have reported that the downregulation of 28 annotated circRNAs, including circ_KIAA0922, characterizes high-cholesterol diet–induced nonalcoholic fatty liver disease (*16*). However, there have not yet been reports on the role of circ_KIAA0922 in bone differentiation.

MicroRNAs (miRNAs) are a type of noncoding RNAs with a regulatory function and a length of approximately 20–25 nucleotides, which identify target mRNAs according to the principle of complementary base pairing and guide the silencing complex to degrade the target mRNAs or repress the translation of mRNAs (*17*). In addition to circRNAs, osteoclast differentiation involves dysregulation and function of miRNAs, such as miR-7223-5p (*18*), miR-19b (*19*), and miR-125b (*20*). Liu *et al*. have demonstrated the inhibitory effect of miR-148a-3p on osteoblast differentiation and bone remodeling (*21*). However, it remains unknown whether miR-148a-3p mediates the function of circ_KIAA0922 in OP.

SMAD5 is an intracellular signal transducer for the transforming growth factor-β (TGF-β) superfamily, which is classified into TGF-βs, activins, and bone morphogenetic proteins (BMPs). As one of the receptor-activated Smad molecules (R-Smad), Smad5 is directly phosphorylated by activated type I TGF-β receptors and associates with the common Smad, Smad4, to form a heteromeric complex, which moves to the nucleus to act as a transcription factor. Many studies have demonstrated that bone morphogenetic proteins (BMPs) and TGF-β are the most important cytokines affecting the differentiation of osteoblasts. BMP-2 is a member of the TGF-β superfamily that is a key signaling component in osteoblast differentiation Smad5 is a *downs*tream transcription factor that is phosphorylated and activated by BMP-2 receptors. SMAD5 is an important element involved in the TGF-β signaling pathway. Activated SMAD5 stimulates the TGF-β signaling pathw*ay*. According to Wang *et al*., miR-148a and SMAD5 participate in the regulation of pancreatic stellate cell activation through a mechanism that involves competing endogenous RNA (*17*).

In the present study, SMAD5 was considered the candidate target gene regulated by miRNA-148a-3p. The role of circ_KIAA0922 in osteogenic differentiation of Saos-2 cells *via* the miRNA-148a-3p/SMAD5/TGF-β axis is also worth further investigation. Taken together, our study aimed to investigate the effects of circ_KIAA0922 on the proliferation and osteogenic differentiation of cultured Saos-2 cells and to clarify its regulatory effects on miR-148a-3p.

2. Materials and Methods

2.1. Cell culture and osteogenic induction

The human osteosarcoma cell line (Saos-2 cells) was purchased from the cell bank of the Chinese Academy of Science (Shanghai, China). Saos-2 cells were cultured in McCoy's 5A medium (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 1% penicillin–streptomycin (Beyotime Biotechnology, Shanghai, China) at 37°C in an atmosphere containing 5% CO_2. The culture medium was changed every two days. For subcultures, the cells at 80–90% confluence were passaged at a ratio of 1:2 using 0.25% trypsin (Beyotime Biotechnology, Shanghai, China). When the cells again grew to 80–90% confluence, they were exposed to McCoy's 5A complete medium containing 2% FBS. The cells were subjected to cell proliferation assay and transfection.

When Saos-2 cells reached 80% confluence, they were cultured in osteoblast medium (OM) supplemented with 10% FBS, 200 μM L-ascorbic acid (Sangon, Shanghai, China), 10 mM glycerophosphate (Sigma-Aldrich, St. Louis, MO, USA), and 100 nM dexamethasone (Sangon) to induce osteoblastic differentiation. The osteogenic induction medium was changed every 3 days, and the cells were collected after 0, 3, 7, and 14 days of differentiation. Saos-2 cells cultured in the growth medium (GM) were used as controls.

2.2. Cell transfection

Small interfering RNA (siRNA) against circ_KIAA0922 (si-circ_KIAA0922) or circ_KIAA0922 overexpression vector (circ_KIAA0922) was synthesized to knock down or elevate circ_KIAA0922 expression, respectively, with si-NC or pCD2.1-ciR utilized as control. The mimic or inhibitor of miR-148a-3p (miR-148a-3p or anti-miR-148a-3p) was used to increase or decrease miR-148a-3p expression, respectively, while miR-NC or anti-miR-NC was used as scramble control. Moreover, siRNA targeting SMAD5 or SMAD5 overexpression was employed to reduce or enhance SMAD5 expression, respectively, while si-control or pcDNA was used as control. All of the above vectors or oligonucleotides were synthesized by GenePharma Co., Ltd. (Shanghai, China). Cell transfection was executed using Lipofectamine 2000

(Invitrogen, Carlsbad, CA, USA) in accordance with the described protocols.

2.3. Cell counting kit-8

Cell counting kit-8 (CCK-8; Beyotime Biotechnology, Shanghai, China) assay was performed to determine the viability of Saos-2 cells. A total of 2×10^3 cells were seeded into 96-well cell culture plates and maintained at 37°C. At each of the desired time points, the plates were cultured for 4 h after adding 10 μL CCK-8 reagent to each well. Next, the wavelength at 450 nm was determined using a microplate reader (Bio-Rad Laboratories, Richmond, CA, USA).

2.4. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA from Saos-2 cells was extracted by TRIzol RNA reagent (Invitrogen, Carlsbad, CA, USA), and 1 μg of RNA sample was prepared for reverse transcription into cDNA using PrimeScriptRT kit (TaKaRa, Shiga, Japan). With cDNA as the template, the noncoding RNA and gene expression levels were analyzed by PCR using SYBR qPCR Master Mix (Sparkjade, Jinan, China) on the Applied Biosystems 7500 Real-time Fast PCR System. By using the $2^{-\Delta\Delta Ct}$ method, the expression was computed with GAPDH or U6 as the internal control. The primers used are presented in Table 1.

2.5. RNase R treatment

To estimate the stability of circ_KIAA0922, the RNA in Saos-2 cells was treated with or without (Mock) RNase R (Epicenter Biotechnologies, Madison, WI, USA) at 37°C for 30 min. Following RNase R treatment, qRT-PCR was performed to detect the expression levels of circ_KIAA0922 and GAPDH.

2.6. Isolation of nuclear and cytoplasmic fractions

Table 1. Primers sequences used for qRT-PCR

Name of primer		Sequences (5'-3')
hsa-circ-KIAA0922	Forward	ACAAAGCCTTCTTCAGAAAAGA
	Reverse	GTGCTTGTTGGAAGGCTATTAAA
OCN	Forward	CTGACCTCACAGATGCCAAGC
	Reverse	TGGTCTGATAGCTCGTCACAAG
RUNX2	Forward	AGCAAGGTTCAACGATCTGAGAT
	Reverse	TTTGTGAAGACGGTTATGGTCAA
hsa-miR-148a-3p	Forward	TCAGTGCACTACAGAACTTTGT
	Reverse	GAATACCTCGGACCCTGC
SMAD5	Forward	CCAGCAGTAAAGCGATTGTTGG
	Reverse	GGGGTAAGCCTTTTCTGTGAG
U6	Forward	GCTTCGGCAGCACATATACTAA
	Reverse	AACGCTTCACGAATTTGCGT
hsa-GAPDH	Forward	GGTGTTCAGCACCCTCTACC
	Reverse	TGTAGCACTTGGCTTTGGAG

In brief, 1×10^6 cultured Saos-2 cells were rinsed in phosphate-buffered saline (PBS) and placed into the cell fractionation buffer and cell disruption buffer in sequence. The fractions of cell cytoplasm or cell nucleus were individually separated in line with the manual of PARIS™ kit (Invitrogen). The expression levels of circ_KIAA0922, U6, and GAPDH were analyzed by qRT-PCR.

2.7. Western blot assay

Total protein in Saos-2 cells was isolated by RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) and quantified utilizing a BCA protein quantification kit (Vazyme, Nanjing, China). Then, the proteins were separated by 10% SDS-PAGE electrophoresis and subsequently transferred onto PVDF membranes (Invitrogen, Carlsbad, CA). The membranes were blocked with 5% defatted milk for 1 h and incubated with primary antibodies against runt-related transcription factor 2 (RUNX2) (bs-1134R; 1:2000; Bioss, Beijing, China), osteocalcin (OCN) (bs-4917R; 1:2000; Bioss), SMAD5 (ab40771; 1:20000; Abcam), transforming growth factor beta 1(TGFβ1) (bs-0086R; 1:1500; Bioss), transforming growth factor beta 2 (TGFβ2) (bs-20412R; 1:1500; Bioss), bone morphogenetic protein 4 (BMP4) (bs-1374R; 1:2000; Bioss) and GAPDH (ab9485; 1:10000; Abcam) at 4°C overnight. After that, the membranes were probed with horseradish peroxidase–conjugated secondary antibody (bs-40296G-HRP; 1:10,000; Bioss) for 1 h at room temperature. The protein bands were visualized *via* ECL kit (Vazyme, Nanjing, China) and quantified using ImageJ software.

2.8. Alizarin Red-S (ARS) staining assay

To detect calcium deposition in the extracellular matrix, Saos-2 cells were subjected to 3, 7, and 14 days of incubation in the osteogenic medium in 24-well plates. After fixation, calcified nodules were treated with 0.1% Alizarin red S solution (Beyo time, Shanghai, China) at pH 4.2. Images were obtained using a microscope (Olympus, Japan). Cetylpyridine chloride (CPC, 100 mM) was used to dissolve calcified nodules, and the relative calcium mass was calculated based on the absorbance at 562 nm.

2.9. Dual-luciferase reporter assay

We used online bioinformatics software (circBank and StarBase) to predict the targets of circ_KIAA0922 and miR-148a-3p. The results showed that miR-148a-3p could potentially be targeted by circ_KIAA0922 and that SMAD5 was a larvaceous target of miR-148a-3p. The circ_KIAA0922 or SMAD5 3'UTR fragment containing the wild-type (WT) or mutant-type (MUT) binding site of miR-148a-3p was cloned into pmirGLO (Geneseed,

Guangzhou, China) to obtain circ_KIAA0922, MUT-circ_KIAA0922, WT-SMAD5 3'UTR, and MUT-SMAD5 3'UTR. The generated vector was cotransfected with miR-148a-3p/miR-NC into Saos-2 cells. At length, a Dual-Luciferase Reporter Assay System (Vazyme, Nanjing, China) was prepared for detecting luciferase vectors at 48 h after transfection. At least three biological replicates were performed to compare luciferase activity between the groups.

2.10. RNA immunoprecipitation assay

The EZMagna RIP-Kit (Millipore Burlington, MA, USA) was used in accordance with the protocols. Target cells were lysed in a complete RNA immunoprecipitation (RIP) lysis buffer, and the cell extract was incubated with magnetic beads conjugated with anti-Argonaute 2 (AGO2) or control anti-IgG antibody (Millipore) for 6 h at 4°C, followed by incubation with Proteinase K to remove the proteins. Purified RNA was analyzed using real-time PCR. Next, enrichment analysis of circ _ KIAA0922, miR-148a-3p, and SMAD5 was performed.

2.11. RNA pull-down assay

Magnetic beads (Thermo Fisher Scientific, Waltham, MA) were incubated with a biotin-labeled miR-148a-3p probe (bio-miR-148a-3p; RiboBio, Guangzhou, China) and a bio-negative control probe (bio-miR-NC; RiboBio) at 4°C overnight. Saos-2 cells were lysed, and the cell lysates were collected and incubated with magnetic beads. Then, the magnetic beads were purified, and the enrichment of circ-KIAA922 and SMAD5 in bio-miR-148a-3p and bio-miR-NC was detected using qRT-PCR.

2.12. Statistical analysis

The results are presented as the mean ± standard error of the mean (SEM). Statistical comparisons were made with one-way ANOVA and the Tukey multiple-comparison tests using GraphPad Prism software, version 7.0 (GraphPad Software Inc., San Diego, CA, USA) to identify significant differences. *P* values lower than 0.05 were considered statistically significant (* represents $P < 0.05$, ** represents $P < 0.01$, *** represents $P < 0.001$). All experiments were performed at least three times.

3. Results

3.1. Characterization and expression analysis of circ_ KIAA0922

Circ_KIAA0922 (chr4: 154524454–154533552), located on chromosome 4, is derived from exon 24 to exon 26 (928 bp) of the host gene KIAA0922 *via* back-splicing. By designing divergent primers and Sanger sequencing, we confirmed the predicted junction of circ_KIAA0922 (Figure 1A). To verify the characterization of circ_ KIAA0922, we first designed specific primers to amplify linear and circ_KIAA0922 sequences. qRT-PCR analysis suggested that circ_KIAA0922 amplification product was only observed in cDNA by divergent primers but not in gDNA (Figure 1B). Then, Oligo (dT)18 primers reverse-transcript cDNA could barely amplify circ_ KIAA0922 compared with Random primers (Figure 1C). In addition, RNase R assay showed that circ_KIAA0922

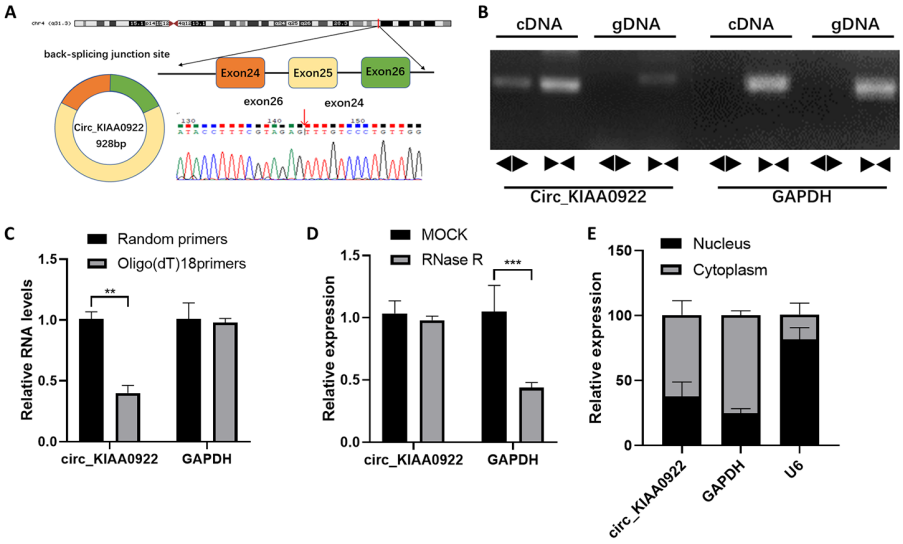

Figure 1. Characterization and expression analysis of circ_KIAA0922. (A) Schematic illustration of the genomic locationand back splicing of circ_KIAA0922 with the splicing site validated by Sanger sequencing. **(B)** RT-PCR with divergent and convergent primers and agarose gel electrophoresis analysis were performeoto detect the presence of circ_KIAA0922 and its maternal gene KIAA0922 in cDNA and gDNA samples from Saos2 cells. **(C)** The RNA in Saos-2 cells was reversely transcribed using Oligo (dT)18 primers or Random primers, and the expression of circ_KIAA0922 and GAPDH was detected. **(D)** RNase treatment was used to evaluate the stability of circ_KIAA0922 and GAPDH mRNA in Saos-2 cells. **(E)** The expression of circ_KIAA0922 in the nucleus and cytoplasm of Saos-2 cells was examined with qRT-PCR.

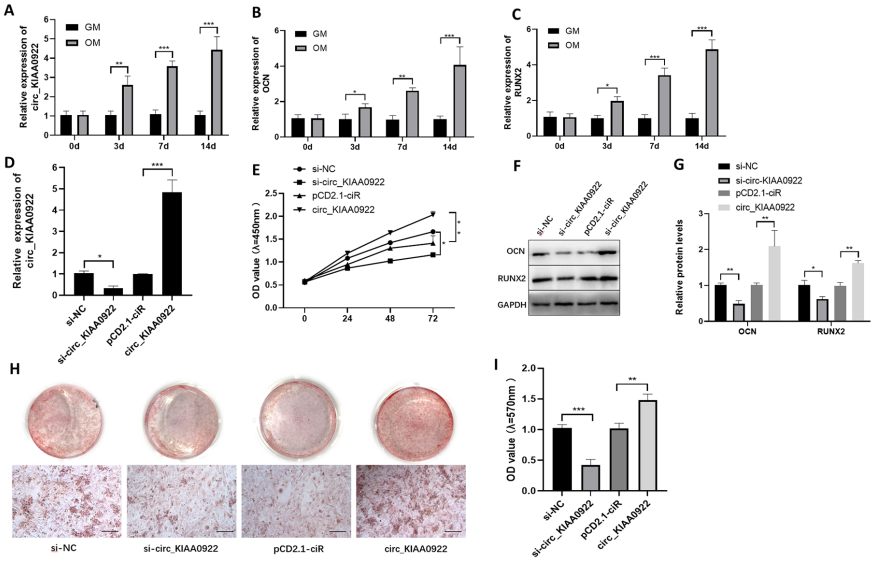

Figure 2. Circ_KIAA0922 contributes to the process of osteogenic differentiation of Saos-2 cells *in vitro*. **(A-C)** The expression of circ_KIAA0922, OCN and RUNX2 in GM- or OM-treated Saos-2 cells was detected by qRT-PCR. **(D-I)** Saos-2 cells were transfected with si-NC, si-circ_KIAA0922, pCD2.1-ciR, or circ_KIAA0922. **(D)** The expression of circ_KIAA0922 in transfected Saos-2 cells was detected by qRT-PCR. **(E)** The proliferation of Saos-2 cells was assessed by CCK-8 assay. **(F** and **G)** The protein levels of OCN and RUNX2 in Saos-2 cells were measured *via* western blot. **(H** and **I)** Alizarin Red staining was used to analyze the calcification of osteogenic differentiation.

was resistant to RNase R treatment, while GAPDH was evidently digested by RNase R (Figure 1D). Subcellular fraction analysis indicated that circ_KIAA0922 was mainly located in the cytoplasm rather than in the nucleus of Saos-2 cells Figure 1E). These findings indicate that circ_KIAA0922 possesses a ring structure and that it is stable.

3.2. Circ_KIAA0922 contributes to the process of osteogenic differentiation of Saos-2 cells *in vitro*

To explore the role of circ_KIAA0922 in the osteogenic differentiation of Saos-2 cells, Saos-2 cells were induced with OM for indicated times, with GM-treated groups used as controls, and transfected with si-circ_KIAA0922 or circ_KIAA0922 overexpression vector to silence or potentiate circ_KIAA0922 expression, respectively. We found that circ_KIAA0922 level after osteogenic induction was markedly increased relative to that in the GM groups (Figure 2A). Next, we detected the expression of the osteogenesis-related markers OCN and RUNX2 in Saos-2 cells treated with GM or OM for 0, 3, 7, and 14 days. We found that OCN and RUNX2 levels were enhanced in OM-induced Saos-2 cells compared with GM-treated Saos-2 cells (Figure 2B-C). All these findings indicated that circ_KIAA0922 might be related to the progression of OP. In addition, si-circ_KIAA0922 transfection markedly suppressed circ_KIAA0922 expression, and circ_KIAA0922 overexpression vector transfection evidently enhanced circ_KIAA0922 expression in Saos-2 cells compared with si-NC or pCD2.1-ciR control groups (Figure 2D). Next, CCK-8 assay was performed to explore the effect of circ_KIAA0922 on the proliferation of Saos-2 cells after 3 days of OM induction. The results showed that circ_KIAA0922 knockdown repressed the proliferation of Saos-2 cells, whereas circ_KIAA0922 overexpression promoted the proliferation of Saos-2 cells (Figure 2E). The results of western blot assay showed that silencing of circ_KIAA0922 decreased the protein levels of OCN and RUNX2 in Saos-2 cells after 3 days of OM induction, while overexpression of circ_KIAA0922 increased OCN and RUNX2 levels (Figure 2F-G). Alizarin Red staining revealed that the matrix mineralization level was decreased by silencing circ_KIAA0922 and increased by elevating circ_KIAA0922 (Figure 2H-I). The elevated OCN level, RUNX2 level, and matrix mineralization level demonstrated the promotional effect of circ_KIAA0922 on osteogenic differentiation of Saos-2 cells *in vitro*.

3.3. Circ_KIAA0922 acts as the sponge for miR-148a-3p

To explore the potential target miRNAs of circ_KIAA0922, the online prediction websites starBase 2.0, circBank and RegRNA 2.0 were used. As displayed in Venn diagram, seven candidate miRNAs (miR-34a-5p, miR-130a-3p, miR-130b-3p, miR-148a-3p, miR-152-3p and miR-145-5p) were found to contain the putative binding sites on circ_KIAA0922 (Figure S1, *http://www.irdrjournal.com/action/getSupplementalData.php?ID=174*). The effect of overexpression or knockdown of circ_KIAA0922 on the expression of the aforementioned miRNA in Saos-2 cells was detected by qRT PCR. The results showed that overexpression of circ_KIAA0922 significantly inhibited the expression of miR-148a-3p and 148b-3p in Saos-2 cells, with the most significant inhibitory effect on miR-148a-3p (Figure S2A, *http://www.irdrjournal.com/action/getSupplementalData.php?ID=174*). In Saos-2 cells, knocking down circ_KIAA0922 significantly promoted the expression of miR-148a-3p and miR-148b-3p. However, the promotion effect on miR-148a-3p is the most significant (Figure S2B, *http://www.irdrjournal.com/action/getSupplementalData.php?ID=174*). Therefore, it is speculated that the circ_KIAA0922

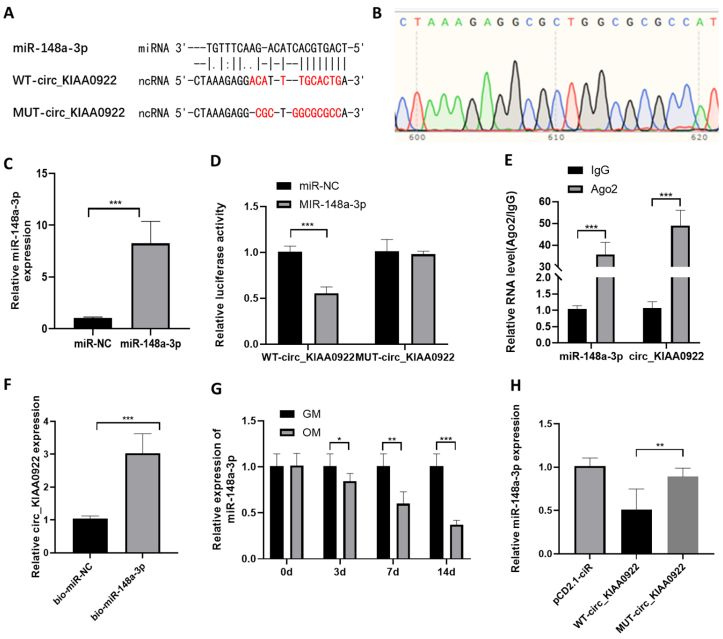

Figure 3. Circ_KIAA0922 acts as the sponge for miR-148a-3p. (A) The binding sites between circ_KIAA0922 and miR-148a-3p. **(B)** DNA sequencing results of mutation vector. **(C)** The expression of miR-148a-3p in Saos-2 cells transfected with miR-NC or miR-148a-3p was detected by qRT-PCR. **(D–F)** The targeting relationship between miR-148a-3p and circ_KIAA0922 was analyzed by dual-luciferase reporter assay, RIP assay, and RNA pull-down assay. **(G)** The expression of miR-148a-3p in Saos-2 cells treated with GM and OM was detected by qRT-PCR. **(H)** The expression of miR-148a-3p in Saos-2 cells transfected with pCD2.1-ciR, WT-circ_KIAA0922, and MUT-circ_KIAA0922 was detected by qRT-PCR.

sequence may contain a miR-148a-3p binding site, which serves as a competitive endogenous RNA in Saos-2 cells and indirectly regulates the expression of downstream target genes of miRNA. Based on the prediction results and previous experimental screening verification, miR-148a-3p was selected for further study. The complementary sequences between circ_KIAA0922 and miR-148a-3p are presented in Figure 3A. DNA sequencing results indicated successful construction of the mutation vector (Figure 3B). The transfection of miR-148a-3p mimic markedly increased miR-148a-3p in Saos-2 cells, compared with miR-NC–transfected cells (Figure 3C). The results of dual-luciferase reporter assay showed that miR-148a-3p overexpression suppressed the luciferase activity of WT-circ_KIAA0922, but it did not affect the luciferase activity of MUT-circ_KIAA0922 (Figure 3D). RIP assay showed that miR-148a-3p and circ_KIAA0922 were evidently increased by Ago2 RIP compared with IgG RIP control (Figure 3E). Moreover, RNA pull-down assay showed that bio-miR-148a-3p pulled down more circ_KIAA0922 than bio-miR-NC (Figure 3F). These results demonstrated the interaction relationship between circ_KIAA0922 and miR-148a-3p. As expected, miR-148a-3p was lowly expressed in OM-induced Saos-2 cells compared with GM-treated Saos-2 cells (Figure 3G). In addition, circ_KIAA0922 overexpression reduced miR-148a-3p expression in Saos-2 cells (Figure 3H). Taken together, circ_KIAA0922 directly interacts with miR-148a-3p.

3.4. circ_KIAA0922 silencing inhibits osteogenic differentiation of Saos-2 cells by targeting miR-148a-3p

As shown in Figure 4A, anti-miR-148a-3p transfection evidently reduced miR-148a-3p expression in Saos-2 cells compared with anti-miR-NC–transfected cells. Next, the relationship between circ_KIAA0922 and miR-148a-3p in regulating the osteogenic differentiation of Saos-2 cells was explored by transfecting Saos-2 cells with si-NC, si-circ_KIAA0922, si-circ_KIAA0922 + anti-miR-NC, or si-circ_KIAA0922 + anti-miR-148a-3p. The qRT-PCR results exhibited that circ_KIAA0922 silencing increased miR-148a-3p expression in Saos-2 cells, whereas the effect was rescued by anti-miR-148a-3p introduction (Figure 4B). CCK-8 assay showed that the inhibitory effect of circ_KIAA0922 knockdown on Saos-2 cells proliferation was rescued by miR-148a-3p inhibition (Figure 4C). As analyzed by western blot assay, circ_KIAA0922 deficiency reduced OCN and RUNX2 protein levels in Saos-2 cells, while miR-148a-3p inhibition reversed these effects (Figure 4D-E). Furthermore, Alizarin Red staining assays showed that the decreased differentiation activity of Saos-2 cells was reversed by downregulating miR-148a-3p (Figure 4F-G). Collectively, miR-148a-3p inhibition reverses circ_KIAA0922 knockdown–mediated suppression of osteogenic differentiation of Saos-2 cells *in vitro*.

3.5. MiR-148a-3p directly targets SMAD5

Subsequently, the potential target genes of miR-148a-3p were explored by analyzing starBase 2.0. We found that SMAD5 contained the binding sites of miR-148a-3p (Figure 5A). Then, dual-luciferase reporter assay, RIP assay, and RNA pull-down assay were conducted to verify the relationship between miR-148a-3p and SMAD5. As indicated by dual-luciferase reporter assay, miR-148a-3p upregulation repressed WT-SMAD5 3'UTR luciferase activity, but MUT-SMAD5 3'UTR luciferase activity was not influenced (Figure 5B). RIP assay results showed that miR-148a-3p and SMAD5 were enriched by Ago RIP compared with IgG RIP (Figure

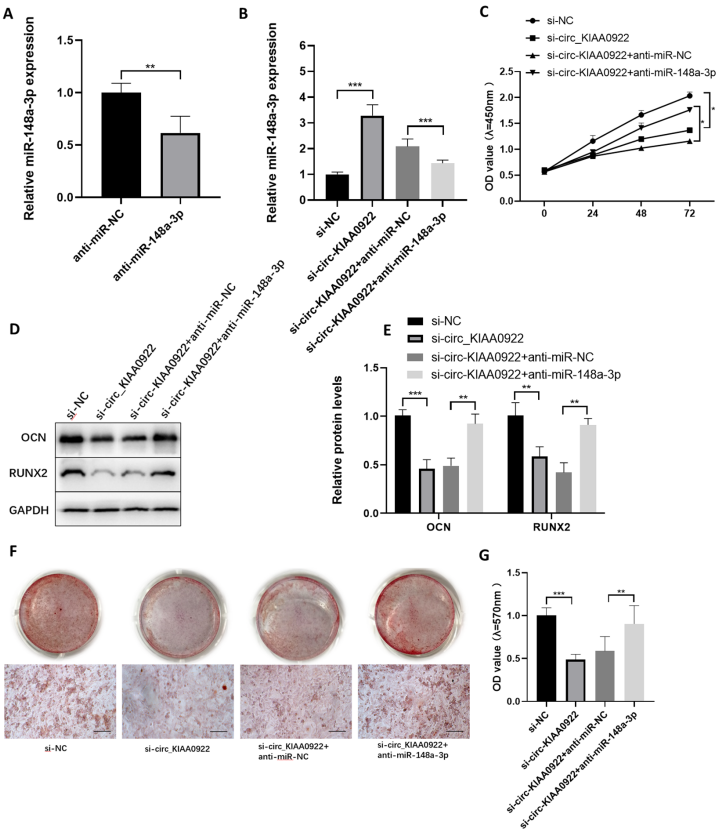

Figure 4. Circ_KIAA0922 silencing inhibits osteogenic differentiation of Saos-2 cells by targeting miR-148a-3p. (A) The expression of miR-148a-3p in Saos-2 cells transfected with anti-miR-NC or anti-miR-148a-3p was detected by qRT-PCR. **(B-E)** Saos-2 cells were transfected with si-NC, si-circ_KIAA0922, si-circ_KIAA0922 + anti-miR-NC, or si-circ_KIAA0922 + anti-miR-148a-3p. **(B)** MiR-148a-3p expression in Saos-2 cells was determined by qRT-PCR. **(C)** The proliferation of Saos-2 cells was evaluated by CCK-8 assay. **(D** and **E)** The protein levels of OCN and RUNX2 in Saos-2 cells were measured by western blot assay. **(F** and **G)** Alizarin Red staining assays were used to determine the ability of osteogenic differentiation of Saos-2 cells. Scale bar, 100 μm.

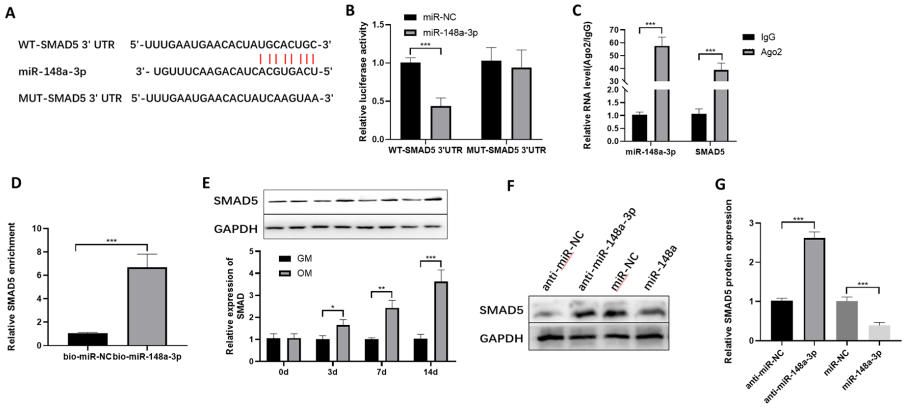

Figure 5. SMAD5 was targeted by miR-148a-3p. (A) The binding sites between SMAD5 and miR-148a-3p were exhibited. **(B-D)** The relationship between miR-148a-3p and SMAD5 was analyzed by dual-luciferase reporter assay, RIP assay, and RNA pull-down assay. **(E)** The protein level of SMAD5 in Saos-2 cells treated with OM or GM was measured by western blot assay. **(F** and **G)** The protein level of SMAD5 in Saos-2 cells transfected with anti-miR-NC, anti-miR-148a-3p, miR NC, or miR-148a-3p was measured by western blot.

5C). The results of RNA pull-down assay suggested that the enrichment of SMAD5 was promoted by bio-miR-148a-3p (Figure 5D). Moreover, SMAD5 protein level was increased in Saos-2 cells after OM induction (Figure 5E). In addition, anti-miR-148a-3p transfection elevated SMAD5 protein level, whereas miR-148a-3p mimic transfection reduced SMAD5 protein level in Saos-2 cells (Figure 5F-G). All these findings suggest that miR-148a-3p interacts with SMAD5 and negatively regulates SMAD5 expression.

3.6. SMAD5 promotes the osteogenic differentiation of Saos-2 cells

To explore the roles of SMAD5 in osteogenic differentiation in Saos-2 cells, Saos-2 cells were transfected with si-SMAD5 or SMAD5. The results of western blot assay showed that si-SMAD5 transfection decreased SMAD5 expression, whereas SMAD5 transfection increased SMAD5 expression compared with relevant controls (Figure 6A-B). The results of CCK-8 assay indicated that SMAD5 knockdown inhibited Saos-2 cells proliferation, whereas SMAD5 overexpression promoted Saos-2 cells proliferation (Figure 6C). Silencing of SMAD5 reduced the protein levels of OCN and RUNX2 in Saos-2 cells, while overexpression of SMAD5 led to the opposite

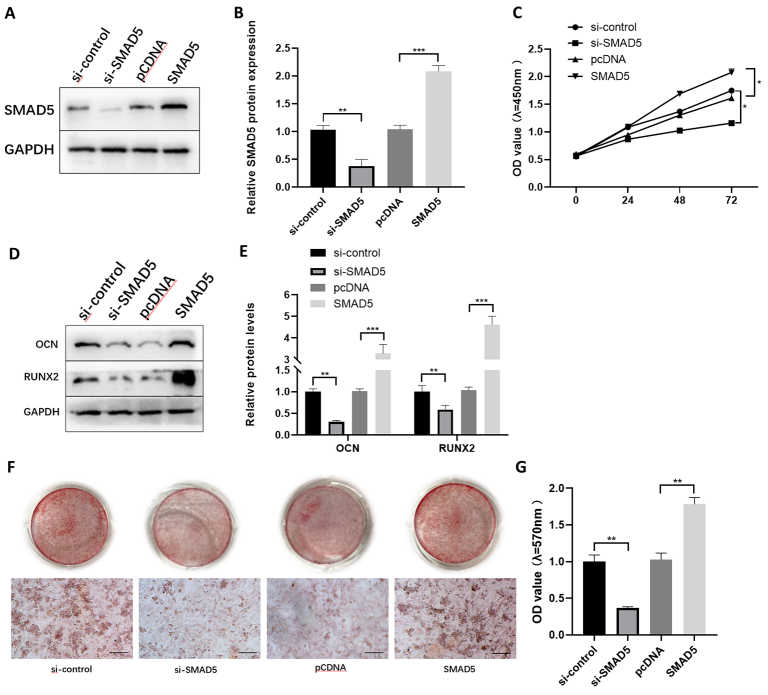

Figure 6. SMAD5 promotes the osteogenic differentiation of Saos-2 cells. Saos-2 cells were transfected with si-control, si-SMAD5, pcDNA, or SMAD5. **(A** and **B)** The protein level of SMAD5 in Saos-2 cells was measured via western blot. **(C)** The proliferation of Saos-2 cells was assessed by CCK-8 assay. **(D** and **E)** The protein levels of OCN and RUNX2 in Saos-2 cells were measured by western blot. **(F** and **G)** The osteogenic differentiation of Saos-2 cells was examined by Alizarin Red staining.

results (Figure 6D-E). As suggested by Alizarin Red staining, SMAD5 knockdown repressed osteogenic differentiation of Saos-2 cells, while SMAD5 overexpression promoted osteogenic differentiation of Saos-2 cells (Figure 6F-G). In summary, SMAD5 overexpression promotes osteogenic differentiation of Saos-2 cells *in vitro*.

3.7. MiR-148a-3p directly targets SMAD5 to regulate osteogenic differentiation of Saos-2 cells

As shown in Figure 7A-B, miR-148a-3p mimic transfection inhibited SMAD5 protein level in Saos-2 cells, but the effect was rescued by the introduction of the SMAD5 overexpression vector. CCK-8 assay indicated that the proliferation of Saos-2 cells was repressed by miR-148a-3p overexpression, but the effect was weakened by elevating SMAD5 (Figure 7C). Overexpression of miR-148a-3p decreased the protein levels of OCN and RUNX2 in Saos-2 cells, whereas SMAD5 upregulation abrogated these effects (Figure 7D-E). Moreover, based on Alizarin Red staining, miR-148a-3p overexpression suppressed osteogenic differentiation of Saos-2 cells, whereas SMAD5 enhancement moderated the effect (Figure 7F-G). Circ_KIAA0922 knockdown reduced the mRNA and protein levels of SMAD5 in Saos-2 cells, while miR-148a-3p inhibition reversed these effects (Figure 7H-G). All these findings suggest that SMAD5 overexpression weakens miR-148a-3p overexpression–mediated inhibition of osteogenic differentiation of Saos-2 cells *in vitro*.

3.8. circ_KIAA0922 targets miR-148a-3p/SMAD5 to regulate osteogenic differentiation and activates the TGF-β signaling pathway in Saos-2 cells

As shown in Figure 8A-B, the proteins involved in the TGF-β signaling pathway, namely, TGF-β1, TGF-β2, and BMP4, were downregulated after miR-148a-3p overexpression, but upregulated after miR-148a-3p downregulation in Saos-2 cells. The promotive effect of miR-148a-3p overexpression on the protein expression levels was rescued by simultaneously overexpressing SMAD5 or circ_KIAA0922. Therefore, the TGF-β signaling pathway in Saos-2 cells was activated by the upregulation of circ_KIAA0922, which resulted in the downregulation of miR-148a-3p and the overexpression of SMAD5. The RNA-induced silencing complex (RISC) led by miR-148a-3p degraded SMAD5 mRNA, while circ_KIAA0922 bound miR-148a-3p to reduce the degradation of SMAD5 (Figure 8C). circ_KIAA0922 upregulated SMAD5 and TGF-β signaling pathway in Saos-2 cells through targeting miR-148a-3p, thereby influencing the progression of osteogenic differentiation of Saos-2 cells.

4. Discussion

OP is a bone metabolic disorder characterized by decreased bone density and deteriorated microstructure, which increases the risk of fractures. The imbalance between bone formation and bone resorption results in the occurrence and progression of OP. Bone homeostasis in the human body is a dynamic equilibrium that consists of bone formation and bone resorption processes. Imbalance and dysfunction of bone homeostasis are the basis of many skeletal diseases, including OP (Kim

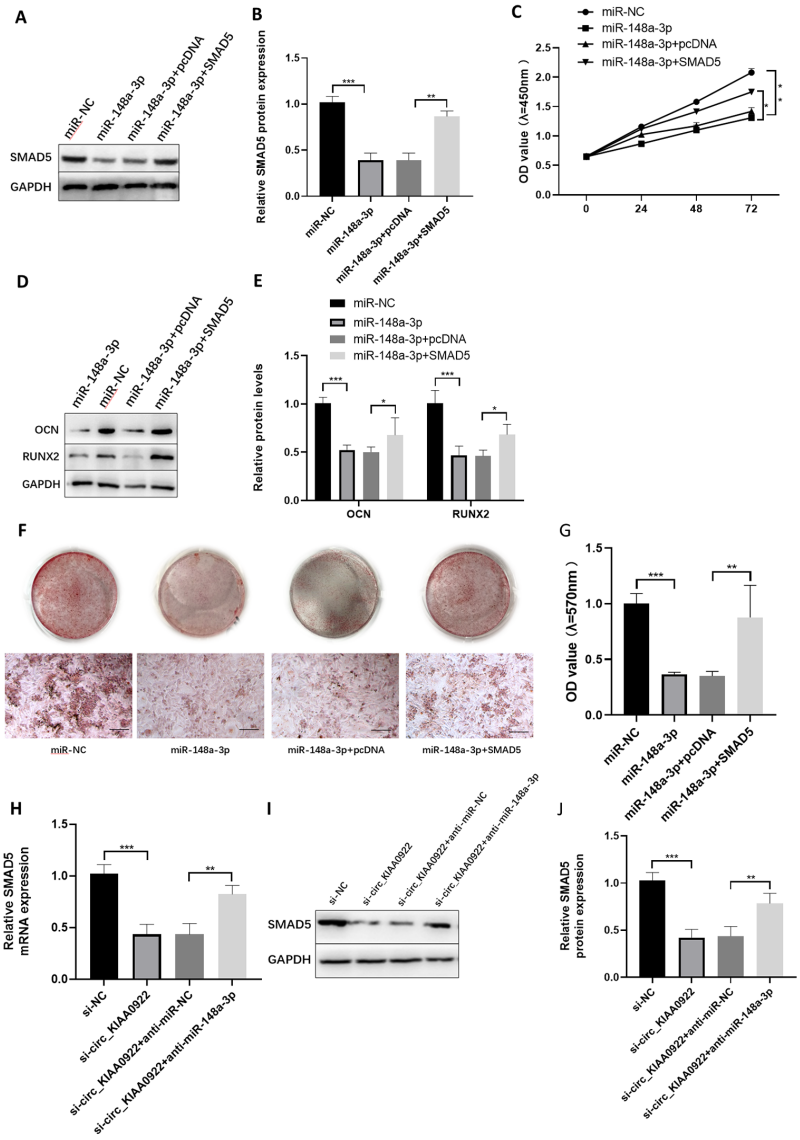

Figure 7. MiR-148a-3p directly targets SMAD5 to regulate osteogenic differentiation of Saos-2 cells. (A-D) Saos-2 cells were transfected with miR-NC, miR-148a-3p, miR-148a-3p + pcDNA or miR-148a-3p + SMAD5. **(A** and **B)** The protein level of SMAD5 in Saos-2 cells was measured via western blot. **(C)** The proliferation of Saos-2 cells was assessed by CCK-8 assay. **(D** and **E)** The protein levels of OCN and RUNX2 in Saos-2 cells were examined by western blot. **(F** and **G)** The osteogenic differentiation of Saos-2 cells was analyzed by Alizarin Red staining. **(H-G)** After Saos-2 cells were transfected with si-NC, si-circ_KIAA0922, si-circ_KIAA0922 + anti-miR-NC, or si-circ_KIAA0922 + anti-miR-148a-3p, the mRNA and protein levels of SMAD5 were determined by qRT-PCR or western blot.

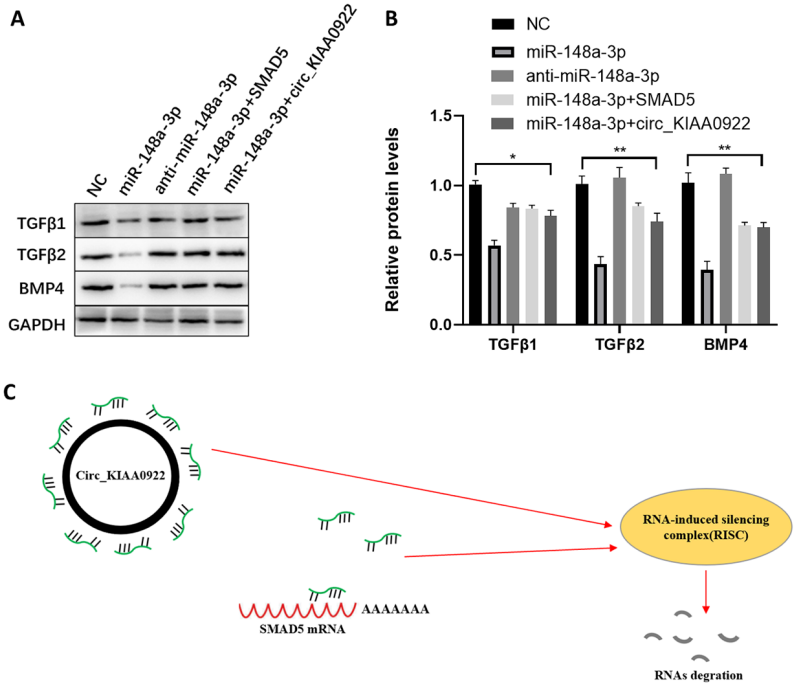

Figure 8. Circ_KIAA0922 targets miR-148a-3p/SMAD5 to regulate osteogenic differentiation and activate the TGF-β signaling pathway in Saos-2 cells. (A and **B)** The relative protein expression levels of TGF-β1, TGF-β2, and BMP4 were decreased in the miR-148a-3p group, increased in the anti-miR-148a-3p group, and rescued in the miR-148a-3p + SMAD5 and miR-148a-3p + circ_KIAA0922 groups. **(C)** The mechanistic diagram illustrates that the RNA-induced silencing complex (RISC) of miR-148a-3p, which degrades the SMAD5 mRNA, would be broken by circ_KIAA0922 binding to miR-148a-3p.

et al., 2020). Bone remodeling is a tightly controlled process in which osteoblasts (the cells responsible for bone formation), osteoclasts (the cells specialized for bone resorption), and osteocytes (the multifunctional mechanosensing cells embedded in the bone matrix) are the main actors. Increased oxidative stress in osteoblasts, the cells that produce and mineralize bone matrix, has been associated with the development of OP.

Saos-2 cells, also known as osteoblast-like cells, are derived from human osteosarcoma and have osteoblastic characteristics in that they can grow, differentiate, and cause mineralization of the extracellular matrix under certain induced conditions, which makes them an important cell line to study osteoblast differentiation (*22*). They are very easily induced to form a large number of mineralized nodules in a short period of time under the action of ascorbic acid and sodium B-glycerophosphate and are also able to express certain osteogenic mineralization signature genes and have a strong osteogenic capacity.

CircRNAs participate in a variety of physiological and pathological processes through diverse mechanisms, including miRNA sponging, interaction with proteins, and transformation into small peptides (*23,24*). It has been suggested that circRNAs may be used as diagnostic biomarkers or treatment targets in OP. For example, Wu *et al*. demonstrated that circRNA_003795 was able to promote the osteogenic differentiation activity of hBMSCs (*25*). Lin *et al*. claimed that the knockdown of circRNA IGSF11 increased miR-199b-5p expression and promoted osteoblast differentiation (*26*). According to Guan *et al*., overexpression of circRNA_0021739 reduces miR-502-5p levels and inhibits osteoclasts differentiation (*27*). Circ-SLC8A1 is downregulated in OP and promotes OP development by miR-516b-5p/AKAP2 (*28*). These reports have indicated the involvement of circRNAs in OP. In this study, we demonstrated that circ_KIAA0922 was upregulated in Saos-2 cells during osteogenic differentiation. Moreover, circ_KIAA0922 knockdown inhibited the proliferation and osteogenic differentiation and reduced the osteogenic differentiation markers (OCN and RUNX2) in Saos-2 cells *in vitro*, indicating the positive regulatory role of circ_KIAA0922 in the osteogenic differentiation of Saos-2 cells. Thereafter, the downstream mechanism underlying the role of circ_KIAA0922 in the osteogenic differentiation of Saos-2 cells was further elucidated.

MicroRNAs are important regulatory factors necessary to maintain bone homeostasis, which is of great significance for bone health and diseases (*29*). Pan *et al*. demonstrated that miR-148a overexpression promoted osteoclastogenesis and bone resorption *in vitro* (*30*), and Liu *et al*. claimed that miR-148a-3p expression was restrained during osteoblast differentiation (*21*). Bedene *et al*. reported that the expression of miR-148a-3p was significantly higher in osteoporotic patients (*31*). In line with these results, we found that miR-148a-3p inhibited osteogenic differentiation of Saos-2 cells *in vitro*. In addition, our findings revealed that circ_KIAA0922 inhibited the proliferation and osteogenic differentiation of Saos-2 cells by targeting miR-148a-3p.

SMAD5 is a critical downstream transcription factor for BMP2. Smad5 phosphorylation due to BMP2 signaling leads to a Smad5 complex formation, which translocates into the nucleus to transactivate the expression of osteogenic genes such as Runx2 and Atf4 (*32*). Previous studies have confirmed its essential role in skeletal development and bone formation (*33*). SMAD5 has been reported to enhance the expression of osteogenic markers, such as OCN and alkaline phosphatase (ALP) (*33*). The active TGF-β signaling pathway can negatively promote osteoblast maturation, mineralization, and osteoblast transformation (*34*). MiRNAs and their targets are mainly involved in the key signaling pathways that control bone formation, including the TGF-β/BMP/SMAD signaling pathways (*5,35*). However, previous studies have not confirmed the relationship between miR-148a-3p and SMAD5 in the process of osteogenic differentiation. In this study, we demonstrated that overexpression of SMAD5 promoted cell proliferation and osteogenic differentiation and increased OCN and RUNX2 levels in Saos-2 cells *in vitro*. Specifically, miR-148a-3p overexpression inhibited the osteogenic differentiation of Saos-2 cells *in vitro* by targeting SMAD5.

In summary, we showed that circ_KIAA0922 affected OP by regulating SMAD5/miR-148a-3p, providing the first line of evidence to clarify the underlying regulatory mechanisms of circ_KIAA0922 in the development of OP *in vitro*. Our findings provide a theoretical foundation for circ_KIAA0922 as a novel diagnostic biomarker and therapeutic target to improve osteogenic differentiation in OP. However, this was an *in vitro* study, and no *in vivo* experiments have been performed, which constitutes a limitation of the present study and calls for additional in-depth investigations in further studies.

Funding: This work was supported by a grant from the Natural Science Foundation of Shandong Province (General program ZR2023MH276) and Academic Promotion Program of Shandong First Medical University (LJ001).

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

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Received August 30, 2023; Revised October 24, 2023; Accepted October 31, 2023.

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Released online in J-STAGE as advance publication November 2, 2023.

Original Article

DOI: 10.5582/irdr.2023.01092

Identification of novel and *de novo GABRB1* mutation in Chinese patient with developmental and epileptic encephalopathy 45

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SUMMARY Developmental and epileptic encephalopathy 45 (DEE45) is an autosomal dominant disease caused by variation in the gamma-aminobutyric acid type A receptor subunit beta 1 (*GABRB1*) gene. Affected individuals have severely impaired intellectual development, hypotonia, and other persistent neurological deficits. However, DEE45 is rare; only four infants with DEE45 have been reported worldwide and no case has been reported in China. Confirming a diagnosis of DEE45 is of great significance for guiding further treatment, assessing patient prognosis, and genetic counseling. The clinical characteristics of DEE45 and the medical history of DEE45 patients requires supplementation and clarification. Here, we present the clinical and genetic findings of a 7-year-old girl with DEE45 carrying a novel *de novo GABRB1* mutation (c.858_859delinsTT, p.286_287delinsIleSer) identified by whole exome sequencing (WES). The mutation is phylogenetic conserved in the second helix of the β1-subunit's transmembrane region. Western blot and RT-qPCR both indicated significant increase in the expression levels of GABRB1 mutant when compared with wild. The proband has epileptic encephalopathy and experienced refractory epilepsy onset at age 2 months and showed developmental delay at age 8 months. Electroencephalography (EEG) displayed hypsarrhythmia. Magnetic resonance imaging (MRI) showed no significant abnormalities in the internal structure of the patient's brain, which is displayed in two previously reported cases. The patient's symptoms of hypotonia, ataxia, profound mental retardation, and dysmetria became evident with development. In summary, we report the genetic and clinical characteristics of the first Chinese patient with DEE45 and explores the relationship between mutation and clinical symptoms.

Keywords DEE45, *GABRB1*, *de novo* mutation, developmental delay, epilepsy of infancy, electroencephalography

1. Introduction

Epileptic encephalopathies include a large group of severe epileptic syndromes with a broad phenotype spectrum. At present, the incidence of epileptic convulsive state, non-convulsive state epilepsy, and sudden unexplained death in epilepsy for these diseases is unknown (*1*). DEE45 is a neurological disorder characterized by significant total developmental retardation in infancy or early childhood and seizures in the first 12 months of life, with frequent mental retardation, functional impairment, and other persistent neurological deficits (*2*). It is caused by a heterozygous variation in the *GABRB1* gene, which encodes a subunit of the gamma-aminobutyric acid type A receptor ($GABA_A$) (*3*). $GABA_A$ is a ligand-gated chloride ion channel that mediates GABAergic inhibition in the central nervous system and is involved in controlling neuronal excitability in the brain and spinal cord (*4,5*). The binding of GABA to a $GABA_A$ receptor can quickly open its CI channel and mediate rapid inhibitory synaptic transmission in the central nervous system (*6*). Changes in the quantity, distribution, and dynamic properties of $GABA_A$ receptors at the synapse are key mechanisms for regulating the intensity of inhibitory synaptic transmission and neural circuit information processing (*7*).

To date, four variations in *GABRB1* have been identified in patients with DEE45, with no patients reported in China. Here, we report the clinical and

genetic findings of a fifth patient with *GABRB1*-related DEE45. This study expands the *GABRB1* variation spectrum and clinical symptoms of this rare disease and contributes to understanding the phenotype genotype relationship of the disease.

2. Subjects and Methods

2.1. Study subjects

This study was approved by the ethics committee of Shandong First Medical University & Shandong Academy of Medical Sciences. We certify that the study was performed in accordance with the Declaration of Helsinki (as revised in 2013). Written informed consent was obtained from the patient's family. Peripheral blood samples were collected from the patient and her parents. The patient's medical records, clinical examination and treatment were reviewed.

2.2. Genetic analysis by WES and Sanger sequencing

Genomic DNA was extracted from blood samples using the TIANamp Blood DNA Kit (TIANGEN Biotech Co., Beijing, China). WES of the proband was performed on the Illumina Novaseq 6000 platform (Illumina Inc., San Diego, CA, USA). After sequencing, base-call file conversion and demultiplexing were conducted using bcl2fastq software (Illumina). The resulting fastq data were analyzed by in-house quality control software to remove low quality reads, and were then aligned to the reference human genome (hs37d5) using the Burrows-Wheeler Aligner (BWA) (*8*), and duplicate reads were marked using Sambamba tools (*9*). Single nucleotide variants (SNVs) and indels were called with GATK (*10*). Copy number variant (CNV) data were detected using the SVD-ZRPKM algorithm, CoNIFER (version 0.2.2) (*11*). Mutation Taster was used for predication of mutation effect (*12*).

After filtering for rare variants, the pathogenicity of identified disease-attributable gene variants was evaluated using the updated guidelines for the interpretation of molecular sequencing from the American College of Medical Genetics and Genomics. The candidate variants were validated in the proband and her parents by Sanger sequencing, which was conducted by Beijing Liuhe BGI.

2.3. Phylogenetic analyses and Three-dimensional (3D) structural model predication

Amino acid sequences among human α1-6, β1-3, and γ1-3 GABAA receptor subunits and amino acid sequences of GABRB1 from ten different species were drawn from National Center for Biotechnology Information website and then aligned using software of Molecular Evolutionary Genetics Analysis (MEGA) and GeneDoc with default setting (*13*). The phylogenetic tree was drawn using MEGA software. Three-dimensional structural model of variant was predicted by AlphaFold Protein Structure Database (*14*).

2.4. *In vitro* expression of *GABRB1*

To explore the effect of the identified *GABRB1* variation (858_859delinsTT) on the expression of *GABRB1*, human embryonic kidney 293 (HEK293) cells were transfected with plasmids of pcDNA3.1 containing wild-type *GABRB1* (WT- *GABRB1*) or variant *GABRB1* (MUT-*GABRB1*) plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to manufacturer's guide, respectively. RT-qPCR and Western blot analysis validated the expression of both mRNA and protein after 72h transfection.

Total RNA from HEK293 cells was extracted using TRIzol RNA reagent (Invitrogen), and 1 μg of RNA was prepared for reverse-transcription into cDNA using a PrimeScriptRT kit (TaKaRa, Shiga, Japan). The expression of *GABRB1* mRNA were analyzed by qPCR using the cDNA as template and SYBR qPCR Master Mix (Sparkjade, Jinan, China) on a Roche 480 Real-time Fast PCR System. The $2^{-\Delta\Delta Ct}$ method was performed to determine expression, with *GAPDH* as the internal control. The primers used in the qPCR are presented in Supplemental Table S1 (*http://www.irdrjournal.com/action/getSupplementalData.php?ID=175*).

Total protein was isolated from HEK293 cells in RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) and quantified using a BCA Protein Quantification Kit (Vazyme, Nanjing, China). The proteins were then separated by 10% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred onto polyvinylidene fluoride membranes (Invitrogen). The membranes were blocked with 5% skimmed milk for 1 h and then incubated overnight at 4°C with primary antibodies against GABRB1 (ab16703; 1:1000; Abcam) and GAPDH (ab9485; 1:10000; Abcam). The membranes were then probed with a horseradish peroxidase conjugated secondary antibody (bs-40296G-HRP; 1:10,000; Bioss) for 1 h at room temperature. The protein bands were visualized using an ECL kit (Vazyme) and quantified using ImageJ software.

2.5. Statistical analysis

The results are presented as the mean ± standard error of the mean. Statistical comparisons were made with one-way ANOVA and the Tukey multiple-comparison tests using GraphPad Prism software, version 7.0 (GraphPad Software Inc., San Diego, CA, USA) to identify significant differences. *P* values lower than 0.05 were considered statistically significant (* represents $P < 0.05$, ** represents $P < 0.01$). All experiments were performed at least three times.

3. Results

3.1. Clinical history of the proband

The proband was a 7-year-old girl of normal height and weight at birth. She was the first child of a non-consanguineous couple of Chinese ancestry. Her family history was unremarkable. The child was born at 41+1 weeks gestation by vaginal delivery. The birth weight was 3,800 g, and the Apgar score was 10 at 0 min, 10 at 5 min and 10 at 10 min according to the standard (the reference population was Asian and Pacific Islander from the National Institute of Child Health and Human Development Fetal Growth Study) (*15*).

The patient had suffered from refractory epilepsy and developmental delay since the age of 2 months. She experienced recurrent convulsions 2 months after birth, characterized by loss of consciousness, clenched teeth, both eyes rolling or staring, facial cyanosis, limb shaking or stiffness, and urinary and fecal incontinence. Pinching therapy had a relieving effect on the condition in the initial stage. An initial diagnosis of hypocalcemia convulsion was made and she received calcium supplementation treatment, but there was no significant improvement in seizure control. Physical examination at the initial diagnosis showed clear consciousness and good mental health. The girl's head was free from deformities and she was sensitive to light. The patient had no resistance in the neck but muscle strength and tension of the limbs were normal. Biochemical examination was generally normal. EEG showed abnormalities with partial electrical seizures originating from the right central and middle temporal regions (Figure 1A).

When the patient was 5 months old, she experienced epileptic relapses with frequent blinking, gnathospasmus, purple lips, clenched hands, and an inability to exhale. Treatment with levetiracetam was initiated, with no improvement in seizure control. Consequently, several different anti-epileptic drugs were administered, including phenobarbital, lysine inositol, vitamin B12, and vitamin B6. Laboratory investigations, including humoral immunity, liver and kidney function, biochemistry, myocardial enzymes, and routine blood parameters, showed hepatic dysfunction. The patient also received new drug treatments, including oxiracetam, ribonucleic acid for injection, and reduced glutathione tablets. EEG examination showed abnormalities, and focal seizures in the right posterior temporal area were detected seven times on waking up and during sleep (Figure 1B). Results from cerebral magnetic resonance imaging (MRI) at the age of 5 months were normal (Figure 1C).

Abnormal EEG was observed at 9 months old. The background brainwaves were mainly characterized by medium to high amplitude irregular fast rhythms, with a high number of high amplitude spike waves, spike rhythms, and delta rhythms distributed asynchronously and asymmetrically in the left and right hemispheres of the brain (Figure 1D). EEG examination at 27 months of age showed an asymmetric frontal background rhythm (Figure 1E). EEG details at different ages are summarized in Supplemental Table S2 (*http://www.irdrjournal.com/action/getSupplementalData.php?ID=175*). Assessment of development at 27 months of age showed that the developmental age of each functional area of the patient was significantly lower than the actual age, especially in terms of adaptability, fine motor skills, and personal social interaction. Their developmental quotient was severely lower than the normal level (Table 1).

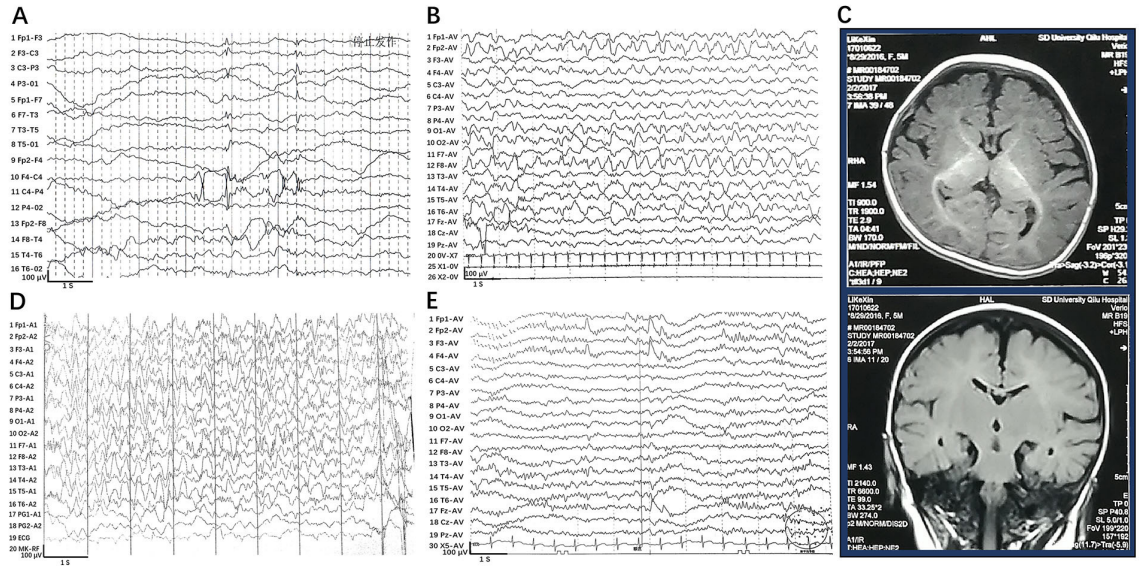

Figure 1. EEG and brain MRI features. (**A**) EEG of the patient at age 2 months was abnormal, with partial electrical seizures originating from the right central and middle temporal regions. (**B**) EEG of the patient at age 5 months showed focal seizures in the right posterior temporal area. (**C**) MRI of the patient at the age of 5 months showed no abnormalities in the brain. (**D**) EEG performed at the age of 9 months showed high amplitude spike waves and spike rhythms. (**E**) EEG of patient at age 27 months showed an asymmetric frontal background rhythm.

At the most recent follow-up, the patient was 7 years old, 115 cm tall, and weighed 17 kg. The patient's symptoms were comprehensive developmental delay, with severe language and motor developmental delay, poor cognitive ability, ataxia, dyskinesia, and decreased overall muscle tone.

3.2. Identification and verification of *GABRB1* mutation

Four variants were identified in the proband by WES: *GABRB1* c.858_859delinsTT, p.286_287delIleSer (NM_000812.4); *KCNQ2* c.1764-5G>A, (NM_172107.4); *KIF7* c.175G>A, p.Val59Met (NM_198525.3); *KIF7* c.3964T>C, p.Ser1322Pro (NM_198525.3). Sanger sequencing confirmed that only the *GABRB1* variation (c.858_859delinsTT) was *de novo* (Figure 2). The other three variants were either found in the proband's mother or father. *GABRB1* c.858_859delinsTT was only identified in the proband and not in her parents.

The p.286_287delIleSer change in *GABRB1* results in replacement of the encoded amino acids, 286 methionine and 287 threonine, with isoleucine and serine, respectively. *In silico* analysis with Mutation Taster predicted the pathogenicity of the variant to be damaging. The variant was not found in the Human Gene Mutation Database (HGMD), ESP6500siv2_ALL, 1000 person genome (1000g2015aug_ALL) and dbSNP147 databases. The variation was classified as likely pathogenic following the American College of Medical Genetics and Genomics guidelines.

3.3. Multiple sequence alignment and 3D structure predication

Multiple sequence alignment analysis among human α1-6, β1-3, and γ1-3 $GABA_A$ receptor subunits (Figure 3A) showed the conserved residues altered by the *de novo* mutations in this study. The GABRB1 protein sequence from different species were aligned and the variant located at amino acid 286-287 of GABRB1 protein within a highly evolutionary conserved region of the subunit (Figure 2B). Phylogenetic analysis indicating that the GABRB1 is relatively conserved among species ranging from fish to humans (Figure 3C).

The 3D view of the GABRB1 protein is shown in Figure 4A, indicating the location of the identified pathogenic variants. The novel variant, p.286_287delinsIleSer, identified in this study is in the second helix of the β1-subunit's transmembrane region affecting transmembrane domains, which is important for the function of the receptor (Figure 4B).

3.4. Increased GABRB1 expression in HEK293 cells

The expression of both wild-type and mutant GABRB1

Table 1. Developmental tests of the patient at the age of 27 months

Test energy zone	DA (month)	DQ	Evaluation
Adaptability	2.33	9	Extremely severe developmental delay
Gross motor	7.93	29	Severe developmental delay
Fine motor	2.80	10	Extremely severe developmental delay
Language	7.23	27	Severe developmental delay
Personal Social	2.10	8	Extremely severe developmental delay

DA: developmental age; DQ: development quotient. (According to the standards of the National Health Commission of China)

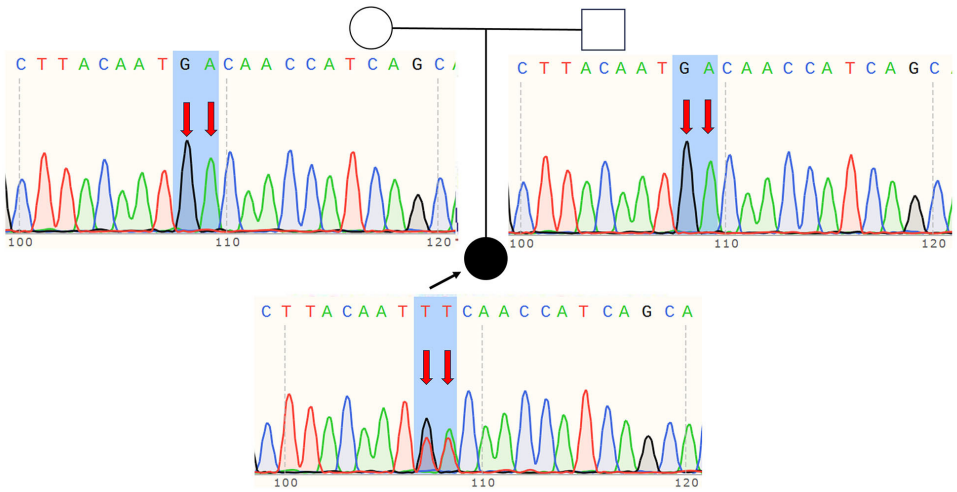

Figure 2. Sanger sequencing results of the proband and her parents. The single-nucleotide substitution is indicated by the red arrow.

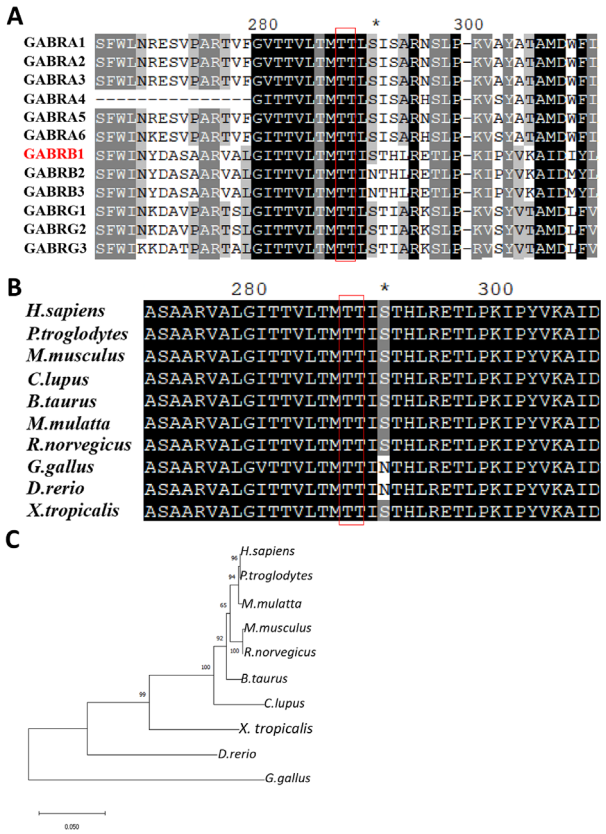

Figure 3. Multiple sequence alignment and phylogenetic analysis of GABRB1. (A) Multiple sequence alignments of human α1-6, β1-3, and γ1-3 $GABA_A$ receptor subunits. The residues highlighted in dark are conserved across all of the subunits. Conserved amino acids are indicated in dark (=100%) and light grey (≧75%) area. **(B)** The variant identified in this study (shown in red box) affected amino acids that are highly conserved from fish to humans. **(C)** Phylogenetic tree of the GABRB1 from different species. Branch confidence levels are built on 1000 bootstrap replicates.

are higher than that of control, referring to the success transfection and expression of wild-type and mutant GABRB1. Significantly enhanced expression was observed in the mutation of p.286_287delIleSer than that of wild-type GABRB1 (Figure 5A). Western blot revealed significant increase of GABRB1 expression level in mutant, which is consistent with the RNA expression (Figure 5B-C). Taken together, these results further confirmed that p.286_287delIleSer variation is a functional mutation. However, the molecular mechanisms still needs to be further explored.

4. Discussion

Epilepsy is a paroxysmal brain disorder that results from an imbalance between neuronal excitation and inhibition. Gamma-aminobutyric acid (GABA) is the most important inhibitory neurotransmitter in the brain and plays an important role in the occurrence and development of epilepsy. Abnormalities in all aspects of GABA metabolism, including GABA synthesis, transport, genes encoding GABA receptors, and GABA inactivation, may lead to epilepsy. *GABRB1* encodes a subunit of one of the GABA receptors and is associated with epilepsy. Heterozygous variations in *GABRB1* on chromosome 4p13 can cause DEE45. Four DEE45 patients with heterozygous variations in *GABRB1* have been identified. In the study, we described a fifth DEE45 patient with a *GABRB1* variation, which is also the first DEE45 case in China.

The Epi4K Consortium and Epilepsy Phenome/Genome Project identified a single *de novo* heterozygous missense variant in *GABRB1* (c.737T>C, p.F246S) in 2013 (*2*). They reported a 4.5-year-old boy with epileptic

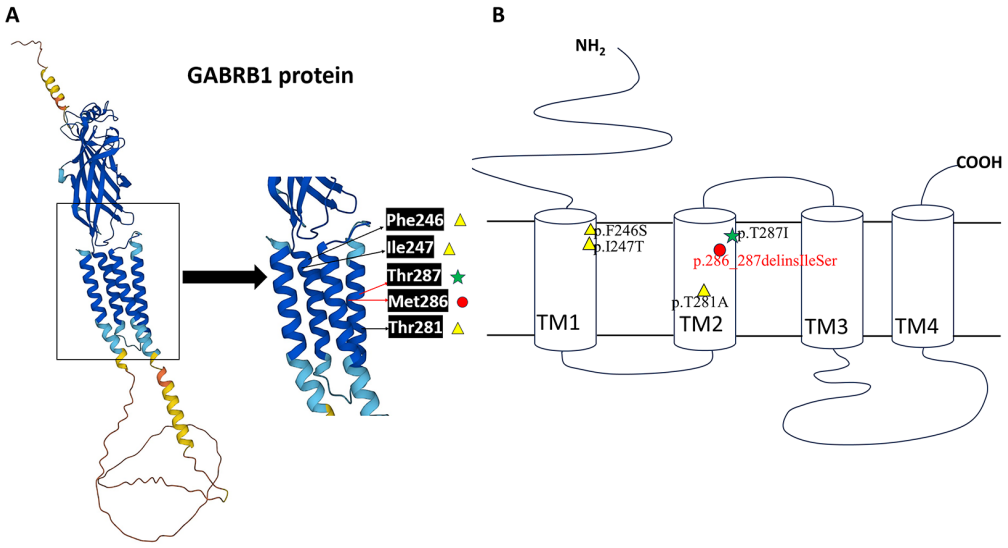

Figure 4. Location of the de novo mutations of *GABRB1* found in DEE45 patients. (A) 3D structural model of the GABRB1 protein (AlphaFold) indicating the positions of the identified pathogenic variants. **(B)** The p.F246S and p.M247I variants were in the TM1 domain; and the p.T281A, p. 286_287delinsIleSer, and p.T287I variants were in TM2 domain. (TM1-TM4) of GABAA receptors were predicted based on the method of Ernst *et al* (*19*). TM, transmembrane-spanning domain. Red circles indicate the variant (p. 286_287delinsIleSer) identified in this study, yellow triangles indicate previously reported variants (p.F246S, p.M247I and p.T281A), and the green star indicates the variants identified in this and previous studies (p. 286_287delinsIleSer and p.T287).

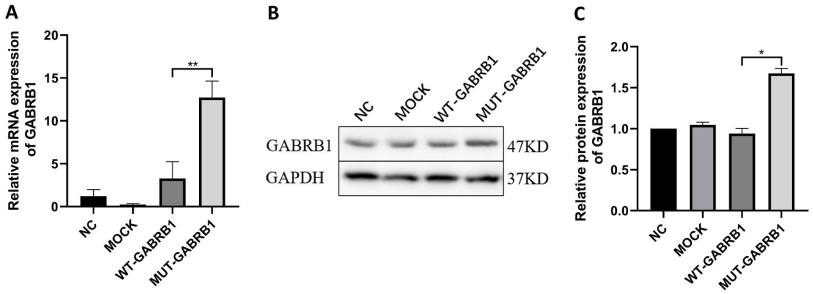

Figure 5. qPCR and Western blot of wild-type and mutant GABRB1 in HEK293 cells. Wild-type (WT) or mutant (MUT) GABRB1 was overexpressed in HEK293 cells. **(A)** mRNA was extracted from cells and quantified using RT-qPCR to measure GABRB1, relative expression of GABRB1 of wild-type is lower than mutant. **(B)** Western blotting showing expression levels of GABRB1 wild-type and mutant. **(C)** The intensity of the bands was quantified by densitometry. Results are expressed as fold-change relative to NC group. Data were normalized to GAPDH. **p* <0.01, ***p* <0.001. NC, negative control; MOCK, transfection reagent control.

encephalopathy. The patient had seizure onset at age 12 months and showed developmental regression at age 35 months. EEG showed hypsarrhythmia. He had global developmental delay, hypotonia, ataxia, cortical visual impairment, and a thin corpus callosum. Janve *et al*. published functional studies on this variant in 2016. The variant is reported to change the kinetic properties of the channel in HEK293 cells, resulting in a net loss of GABAergic inhibition (*16*).

In 2016, Lien *et al*. reported a 32-month-old boy with severe developmental delay and hypotonia who developed refractory epilepsy at age 3 months (*17*). Brain MRI of this patient was normal. This was the second case of DEE45 with a *de novo* heterozygous missense variation in GABRB1 (c.860C>T, p.T287I).

Burgess *et al*. (2019) reported a 2-year-old girl with DEE45 who carried a *de novo* heterozygous missense variation in *GABRB1* (c.740T>C, p.I247T). She experienced the onset of focal and tonic seizures at 4 months of age with cessation of seizures at about 2 years of age. She had profoundly impaired intellectual development with hypotonia, dysmorphic features, and progressive cerebral and white matter atrophy on brain imaging (*1*).

Monfrini *et al*. (2023) described the clinical and genetic findings of a 21-year-old woman with DEE45 carrying a novel *de novo* GABRB1 variation (c.841A>G, p.T281A) which was the fourth report of a DEE45 patient (*18*). This patient presented at birth with hypotonia and focal apneic seizures evolving in a phenotype of epilepsy of infancy with migrating focal seizures that were refractory to anti-seizure medications. Epileptic spasms that were partially responsive to steroid therapy appeared in the second year of life. Acquired microcephaly, profound mental retardation, and tetraparesis became evident with development. During childhood and adolescence, the epileptic phenotype evolved towards Lennox-Gastaut syndrome.

The novel mutation (p.286_287delinsIleSer) reported in our study is localized in the proximity of the previously described *GABRB1* pathogenic variant, p.T287I (Lien *et al*. in 2016), which affects the same alpha-helical transmembrane domain. In addition, methionine and threonine in this position are phylogenetic conserved. All above five *GABRB1* pathogenic variants affect transmembrane domains of the GABRB1 protein, possibility indicating a common molecular pathogenic mechanism causes of DEE45.

More noteworthy, however, is that through comparative analysis of these five DEE45 cases, we found that the brain MRI results of patients with p.F246S, p.I247T and p.T281A variants all showed a thin corpus callosum or white matter lesions. However, the brain MRI of the patient in this study (with a p.286_287delinsIleSer variant) and of a previously reported patient with a p.T287I variant were normal. Moreover, these two patients are similar in terms of age of onset. The overall severity of symptoms is also relatively mild compared with that in patients with the other three variants (p.F246S, p.I247T and p.T281A).

Taken together, this study reports the first case of DEE45 in China. Relationship between genotype and phenotype of DEE45 were overviewed through case analysis. The study also provides further clinical and molecular evidence for exploring the role of GABRB1 in this disease.

Funding: This work was supported by a grant from the Natural Science Foundation of Shandong Province (General program ZR2023MH276) and Academic Promotion Program of Shandong First Medical University (LJ001).

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

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Received October 12, 2023; Revised November 2, 2023; Accepted November 4, 2023.

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Released online in J-STAGE as advance publication November 6, 2023.

Original Article

DOI: 10.5582/irdr.2023.01056

Expression of collagen-related piRNA is dysregulated in cultured dermal fibroblasts derived from patients with scleroderma

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SUMMARY PIWI-interacting RNA (piRNA) is a class of recently discovered small non-coding RNAs. piRNAs derive from an initial transcript encompassing a piRNA cluster via a unique biosynthesis process, interact with PIWI proteins, bind to specific targets, and recruit chromatin modifiers to enable transcriptional repression. Abnormal expression of PIWI proteins and piRNAs has been reported in some human cancers, with participation of some PIWI/piRNAs complexes in tumorigenesis and association with cancer prognosis. Their expression in patients with systemic sclerosis (SSc) has not been widely elucidated. PIWI/piRNAs and their role in the pathogenesis of collagen accumulation in SSc was therefore investigated; no difference was found in the PIWIL1-4 levels between normal and cultured SSc dermal fibroblasts. Among piRNAs predicted to target SSc-related molecules, we first found significant piR-32364 up-regulation in SSc dermal fibroblasts, likely due to intrinsic TGF-β signaling. Forced piR-32364 overexpression in normal fibroblasts significantly reduced COL1A1 expression both at mRNA and protein levels, but not COL1A2. Thus, piR-32364 overexpression in SSc fibroblasts may be the negative feedback against collagen up-regulation, which could suggest the potential of piRNAs as a therapeutic target.

Keywords fibrosis, collagen, piRNA

1. Introduction

Despite the remarkable advances in medical science in recent years, there are still many diseases whose pathogenesis have yet to be unclarified. Among them, systemic sclerosis (SSc) is distinguished by multiple-organ tissue fibrosis, including the skin and lungs. The mechanism of abnormal collagen expression as a cause of the fibrosis is still unknown, and treatments for the disease are sometimes ineffective. Numerous clinical and basic research studies have been conducted, but the disease is heterogeneous, and its manifestation varies in each case.

Genetic and environmental factors could each have involvement in the pathogenesis of collagen diseases, and the latter mainly affecting the heterogeneity. Non-coding RNA has been the focus of our research as an environmental factor. Small non-coding RNAs include microRNAs (miRNAs) and small interfering RNAs (siRNAs) (*1,2*). Mature forms of such RNAs associated with biogenesis pathway proteins include Argonaute protein to guide target gene regulation. Meanwhile, PIWI-interacting RNAs (piRNAs), recently discovered small non-coding RNAs comprising 24–31 nucleotides, have a strong 5′-terminal uridine or tenth position adenosine bias, but lack clear secondary structure motifs (*3,4*). Unlike miRNAs and siRNAs, piRNAs derive from an initial transcript encompassing a piRNA cluster *via* a unique biosynthesis process (*5*), interact with PIWI proteins, bind to specific targets (based on sequence specific complementarity), and recruit chromatin modifiers to enable transcriptional repression (*6*). They were originally believed to control only transposons and development of germinal stem cells, but piRNAs have become known to control epigenetic regulation both at transcriptional and post-transcriptional levels.

Abnormal expression of PIWI proteins and piRNAs has been reported in human cancers, with some PIWI/piRNAs complexes being involved in tumorigenesis and associated with cancer prognosis (*7-9*). Expression in patients with SSc has not been widely elucidated. We therefore investigated the role of PIWI/piRNAs in the

pathogenesis of collagen accumulation in SSc.

2. Materials and Methods

2.1. Cell cultures

This study received approval from the Wakayama Medical University Research Ethics Committee (No. 2446), with written informed consent obtained from all patients. It was conducted in accordance with the Declaration of Helsinki. Human dermal fibroblasts were obtained by skin biopsy from the affected areas (dorsal forearms) of three diffuse cutaneous SSc patients with < 2 years of skin thickening. Control normal dermal fibroblasts were also obtained, as previously described (*10*). Dermal fibroblasts were cultured in Minimum Essential Medium Eagle (Sigma-Aldrich, St Louis, MO) supplemented with 10% fetal bovine serum (EQUITECH-BIO, Kerrville, TX) and Antibiotic-Antimycotic (Gibco, Waltham, MA). Monolayer cultures independently isolated from different individuals were maintained at 37°C in 5% CO_2. Cells were serum-starved before all experiments.

2.2. Skin samples

Skin specimens were obtained from the skin of the involved forearms of patients with SSc (n = 5), and normal skin was also collected for use as controls. These skin samples were fixed in formalin and embedded in paraffin immediately after collection.

2.3. Synthetic oligo

Human dermal fibroblasts were transfected with synthetic piR-32368 oligo (sense; GGTGAAAATGGAGCTCCTGGTCAGATG; antisense; CATCTGACCAGGAGCTC CATTTTCACC) as reported previously (*11*). Scrambled and non-targeting RNA was used as a control (Dharmacon, Lafayette, CO).

2.4. RNA isolation and quantitative real-time polymerase chain reaction (PCR)

Total RNA from cultured fibroblasts were extracted using RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Total RNA from paraffin sections was extracted with RNeasy FFPE Kit (QIAGEN).

cDNA was synthesized from the total RNA with PrimeScript RT Reagent Kit (Takara, Kusatsu, Shiga, Japan). Primers for COL1A1 (forward: GCTTGGTCCACTTGCTTGAAGA, reverse: GAGCATTGCCTTTGATTGCTG), COL1A2 (forward: GAGGGCAACAACAGCAGGTTCACTTA, reverse: TGGGCCAATGTCCACAAAGA), and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward: AGGGCTGCTTTTAACTCTGGT, reverse: CCCCACTTGATTTTGGAGGGA) were purchased from FASMAC. PIWIL gene primers were designed as previously described (*12*). All of these primers have been pre-verified to produce a single amplicon. Quantitative real-time PCR was performed with TaKaRa Thermal Cycler Dice (TP900) using TB Green Premix Taq II (Tli RNaseH Plus) (Takara). Data from each PCR reaction was analyzed by Thermal Cycler Dice real time system ver. 5. 11 (Takara). The transcript levels of each gene were normalized to those of GAPDH.

2.5. Quantitative real-time PCR for piRNA

Mir-X miRNA First-Strand Synthesis Kit (Takara) was used for cDNA synthesis from piRNAs. The sequences of piRNA primers were designed based on piRNAdb (*https://www.pirnadb.org*). The template was amplified and annealed as previous reported (*13,14*). Transcript levels of all piRNAs were normalized to those of U6 in the same sample.

2.6. Cell lysis and immunoblotting

Fibroblasts were cultured until they were confluent, before removal of the medium from the culture dishes. Cells were washed twice with phosphate buffered saline (PBS) and lysed in M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA). Aliquots of the cell lysates were separated by electrophoresis, as previously described (*15*). The primary antibody for type I collagen were purchased from Southern Biotechnology Associates (Birmingham, AL). The immunoreactive bands were visualized using Clarity Western ECL Substrate (Bio-Rad, Hercules, CA).

2.7. Statistical analysis

The bar graphs were the means + standard deviation (SD). Mann-Whitney U test was used for statistical analysis. P values < 0.05 were considered significant.

3. Results

3.1. PIWIL gene expression in cultured SSc dermal fibroblasts

Expression of PIWIL genes were first compared using real-time PCR with RNA extracted from cultured dermal fibroblasts of three SSc patients and three normal subjects. Only PIWIL4 was detected by our protocol (Figure 1A). When the transcription level of PIWIL4 was corrected with that of GAPDH in each sample, PIWIL4 expression in SSc fibroblasts were similar to the normal control cells, with no significant differences.

3.2. piRNA expression in cultured SSc dermal fibroblasts

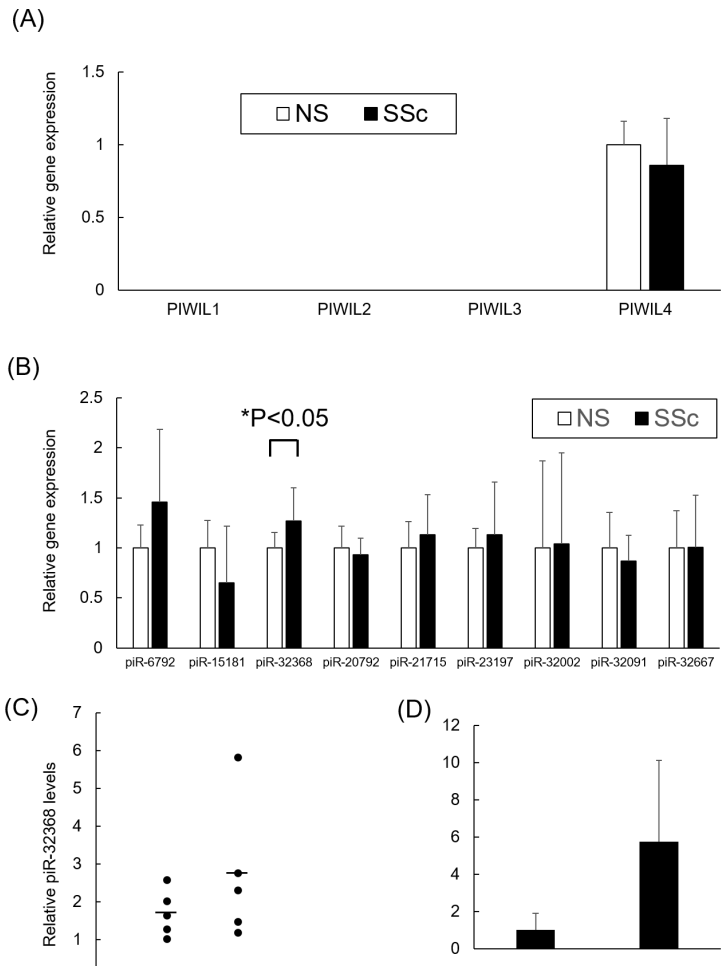

Figure 1. Expression of piR-32368 in the skin fibroblasts and tissues of patients with SSc. (A) Relative PIWI gene expression in cultured dermal fibroblasts derived from SSc patients and normal subjects (NS). Total RNA was extracted, and levels of indicated genes were analyzed by real-time PCR. The transcript level of each gene was normalized to that of GAPDH. The bars represent the mean values, and the T bars indicate standard deviations. The mean value of NS cells was set at 1. **(B)** Relative piRNA expression in cultured dermal fibroblasts derived from SSc patients and normal subjects (NS). RNA was extracted, and indicated piRNA levels were analyzed by real-time PCR. The transcript level of each gene was normalized to that of U6. The bars represent the mean values, and the T bars indicate standard deviations. The mean value of NS cells was set at 1. *$P < 0.05$ ($n = 3$). **(C)** Relative piRNA expression in skin tissues of five SSc patients and of control subjects (cont). RNA was extracted, and mRNA levels of piR-32368 were analyzed by real-time PCR. The lowest value in control tissues was set at 1. **(D)** piR-32368 levels in normal fibroblasts in the presence or absence of TGF-β1 (5 ng/mL) for 3 hours. *$P < 0.05$ compared to untreated normal fibroblasts (1.0).

Next, we compared piRNA expression of normal and SSc dermal fibroblasts. As the key molecules in the pathogenesis of SSc, we focused on COL1A1, COL1A2, COL3A1, KLF5, and Fli1. According to piRNA target gene predictions using piRNAdb (https://www.pirnadb.org), nine piRNAs were selected: has-piR-6792 for COL3A1, piR-15181 for COL1A1 and Fli1, piR-32368 for COL1A1, piR-20792 for Fli1, piR-23197 for Fli1 and KLF5, piR-32002 for Fli1, piR-21715 for Fli1, piR-32091 for COL3A1, and piR-32667 for COL1A2. Quantitative real-time PCR analysis was performed using the specific primer for each piRNA. As a result, among the nine piRNAs, only piR-32368 level was significantly up-regulated in SSc dermal fibroblasts (Figure 1B). Expression of piR-32368 in RNA extracted from five SSc whole skin tissues and normal skin tissues was also investigated: It was slightly increased in SSc skin, but without statistical significance (Figure 1C). piR-32368 up-regulation may therefore be highly specific to fibroblasts, but not in other cell types. To examine the possibility that the up-regulation of piR-32368 in SSc fibroblasts is due to the stimulation of intrinsic transforming growth factor (TGF)-β activation seen in these cell types as described previously (*16*), normal fibroblasts were stimulated with exogenous TGF-β1. TGF-β1 induced piR-32368 expression significantly (Figure 1D), suggesting that up-regulation of piR-32368 is consequence of activation of TGF-β signaling in SSc fibroblasts, at least partly.

3.3. Collagen expression in cultured dermal fibroblasts with transfected piR-32368

To better understand the role of piR-32368 up-regulation in SSc dermal fibroblasts, total RNA was isolated from cultured normal dermal fibroblasts transfected with or without piR-32368 synthetic oligo. Expression analysis showed that the oligo significantly induced the expression of piR-32368, and that piR-32368 overexpression was achieved with transfection of the oligo (Figure 2A). Furthermore, transfection of dermal fibroblasts with the piRNA oligo significantly reduced the expression of COL1A1 both at the mRNA levels (Figure 2B) and protein levels (Figure 2C). However, COL1A2 expression was not affected. These results indicate that COL1A1 is the specific target of piR-32368 in dermal fibroblasts.

3.4. Expression of piR-32368 in the sera of SSc patients

Lastly, we aimed to determine serum piR-32368 levels in patients with SSc and in control subjects using the same

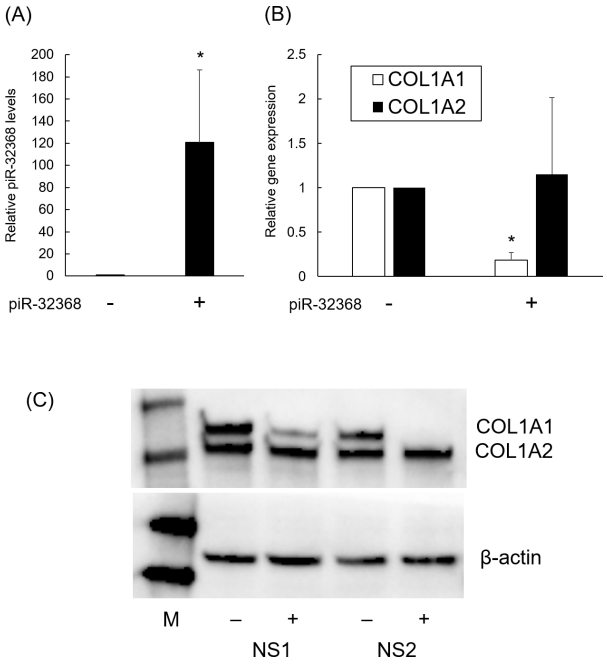

Figure 2. The function of piR-32368 in dermal fibroblasts. (A) Relative gene expression in cultured normal dermal fibroblasts transfected with or without piR-32368 synthetic oligo. RNA was extracted, and mRNA levels of piR-32368 were analyzed by real-time PCR. The mean value of cells transfected with control oligo was set at 1. *$P < 0.05$ ($n = 3$). **(B)** Relative gene expression in cultured normal dermal fibroblasts transfected with or without piR-32368 synthetic oligo. RNA was extracted, and mRNA levels of COL1A1 and COL1A2 were analyzed by real-time PCR. The mean value of cells transfected with control oligo was set at 1. *$P < 0.05$ ($n = 3$). **(C)** Lysates from two different normal dermal fibroblasts treated with or without synthetic oligo for 48 hours were subjected to immunoblotting with antibody against type I collagen or β-actin. M; molecular marker.

method as used in the studies of miRNAs and yRNAs (*14,15*), but amplification of the piRNA in the serum using real-time PCR could not be detected (data not shown).

4. Discussion

piRNAs have been implicated in the pathogenesis of several cancers (*2,4*). In patients with SSc, however, the expression and role of PIWI/piRNA has not been understood. In the current study, PIWIL1-3 was not detected in normal dermal fibroblasts. PIWIL1 and PIWIL2 have been reported to be found in the testes, while PIWIL3 could not be detected in human adult testes but is expressed in fetal testes and ovaries (*17*). The tissue specificity of PIWIL4 seems to be low, and the present study may be the first to demonstrate the expression of PIWIL4 in dermal fibroblasts according to PubMed search using keywords "fibroblast" and "PIWIL4". However, the PIWIL4 levels in normal and cultured SSc dermal fibroblasts were similar.

On the other hand, we focused on COL1A1, COL1A2, COL3A1, KLF5, and Fli1 as the SSc-related molecules, because they were are all reported to be affected by epigenetic regulation in patients with SSc (*18-22*). As far as we are aware base on search by PubMed using keywords "systemic sclerosis", "fibroblast" and "piRNA", we first found significant piR-32368 up-regulation in SSc dermal fibroblasts. There are no known reports on piR-32368, and its role and regulatory mechanism in human diseases remains unknown. Excess collagen production by dermal fibroblasts is thought, at least in part, to be caused by intrinsic activation of TGF-β signaling in SSc (*16*). Our study also indicated that the up-regulation of piR-32368 in SSc fibroblasts may also result from the activated endogenous TGF-β signaling.

SSc is an autoimmune disease in which fibroblasts produce collagen fibers more aggressively than normal. We therefore hypothesized that up-regulation of piR-32368 in SSc dermal fibroblasts stimulates collagen expression. Contrary to the expectation, however, forced piR-32368 overexpression in normal fibroblasts significantly reduced the expression of COL1A1 both at the mRNA levels and protein levels, but not COL1A2. COL1A1 is therefore thought to be the specific target of piR-32368. The vast majority of collagens accumulated in lesional skin of SSc are type I collagen: Type I collagen molecules consists of two α1(I) chains and one α2(I) chain, and COL1A1 and COL1A2 genes are located on chromosomes 17 and 7, respectively. COL1A1 and COL1A2 may therefore be regulated by different mechanisms. COL1A1 expression is, at least in part, regulated by piRNAs, while COL1A2 is unlikely to be targeted by piRNAs because the expression of COL1A2-associated piRNA was not altered in SSc dermal fibroblasts. piRNAs may be a clue to the distinct regulation of COL1A1 and COL1A2. Taken together, piR-32368 itself, which have suppressive effect on COL1A1 production, was up-regulated in SSc dermal fibroblasts. piR-32368 overexpression in SSc fibroblasts may therefore be negative feedback against collagen up-regulation, suggesting that piRNAs could be considered as a potential therapeutic target.

Serum piRNA has been reported to be a novel biomarker in human cancers. Serum exosomal piR-hsa-26925 and piR-hsa-5444 could, for example, serve as potential biomarkers for diagnosis of lung adenocarcinoma (*23*). However, amplification of the piRNA could not be detected in the human serum samples using real-time PCR in the current study. Further studies are needed to detect serum piR-32368 and to evaluate its usefulness in the diagnosis of SSc.

In conclusion, among piRNAs predicted to target SSc-related molecules, we first found significant piR-32368 up-regulation in SSc dermal fibroblasts, likely due to intrinsic TGF-β signaling. Forced piR-32368 overexpression in normal fibroblasts significantly reduced the expression of COL1A1 both at the mRNA levels and protein levels, but not COL1A2. piR-32368 overexpression in SSc fibroblasts may therefore be

negative feedback against collagen up-regulation, so piRNAs could be a potential therapeutic target.

Funding: This study was supported in part by a grant for scientific research from the Japanese Ministry of Education, Science, Sports and Culture. We acknowledge proofreading and editing by Benjamin Phillis at the Clinical Study Support Center, Wakayama Medical University.

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

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Received July 12, 2023; Revised September 25, 2023; Accepted October 16, 2023.

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Released online in J-STAGE as advance publication October 23, 2023.

Brief Report

DOI: 10.5582/irdr.2023.01090

Herpes zoster peripheral nerve complications: Their pathophysiology in spinal ganglia and nerve roots

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SUMMARY Varicella zoster virus (VZV) causes chickenpox at the primary infection and then becomes latent in the spinal dorsal root ganglia; VZV can reactivate with aging, immunosuppression, stress, and other factors, occurring as herpes zoster (HZ) at 1–2 skin segments. HZ peripheral nerve complications caused by VZV reactivation include Hunt syndrome, segmental HZ paresis, post-herpetic neuralgia, and Guillain-Barré syndrome (GBS). We have encountered the rare HZ complications of upper-limb paresis, myeloradiculitis, and polyradiculoneuritis: an adult woman with upper-limb paresis consistent with the nerve root on segments above the thoracic HZ dermatome; another woman exhibiting ascending myeloradiculitis originating at the Th11–12 roots; an elderly woman with ascending VZV polyradiculoneuritis resembling GBS; an adult with VZV quadriplegia with disseminated HZ; and an elderly patient with VZV-associated polyradiculoneuritis. The three polyradiculoneuritis cases may be a new subtype of HZ peripheral neuropathy, but the pathophysiology for these HZ peripheral nerve complications unrelated to HZ dermatomes is unclear. We analyzed host factors, skin lesions, neurological and virological findings, and MRI results including 3D NerveVIEW in 15 Japanese patients treated at our facility for HZ peripheral neuropathy, including six differing from the HZ dermatome. Based on the clinical findings including MRI results of spinal ganglia and roots, we identified four possible routes for the patterns of VZV spread: (*i*) ascending spinal roots, (*ii*) ascending spinal cord, (*iii*) polyradiculopathy, and (*iv*) intrathecal spread. The incidence of HZ is increasing with the aging of many populations, and clinicians should be aware of these HZ neuropathies.

Keywords herpes zoster, varicella-zoster virus, spinal ganglia, neuropathy, segmental paresis, polyradiculoneuritis

1. Introduction

Varicella-zoster virus (VZV) causes varicella as a primary infection, then becomes latent in trigeminal and spinal ganglia. Decades later, herpes zoster (HZ) occurs when the latent VZV spreads to one or two dermatomes in adult and elderly individuals with a suppressed immune system (*1*). There are few pathological findings in the spinal ganglia, but the VZV extends beyond 2–3 spinal ganglia or the anterior horn of the spinal cord and the opposite side (*2,3*). HZ peripheral nerve complications include Hunt syndrome, segmental zoster paresis, Guillain-Barré syndrome (GBS), and post-herpetic neuralgia (PHN) (*4,5*). These HZ complications with radicular pain are intractable and severely impair an individual's activities of daily living and quality of life (*6*).

Since 2018, we have reported the rare HZ peripheral nerve complications of upper-limb zoster paresis, myeloradiculitis, and polyradiculoneuritis. For example, we treated a 53-year-old woman who presented upper-limb paresis corresponding to the nerve root on segments above the thoracic HZ dermatome, and a 32-year-old woman showed ascending myeloradiculitis that originated on HZ Th11–12 roots (*7,8*); in addition, an elderly woman who had an HZ duplex in the right auricle and left shoulder developed right Hunt syndrome, followed by left upper-limb paresis (*9*). We also treated an elderly woman with VZV-associated ascending quadriplegia with HZ rash on the cervical 7–8 regions, with an increase in cerebrospinal fluid (CSF) cells (*10*). An adult patient with VZV polyradiculoneuritis with disseminated HZ and an elderly patient with VZV-

associated polyradiculoneuritis were also treated at our hospital (*11,12*).

These patients' cases shared the characteristic of main lesions in the spinal nerve root, but the pathophysiology for the HZ neurological complications that are unrelated to the HZ dermatomes has not been clarified. In the past 7 years at our hospital, there have been 15 patients with HZ peripheral nerve complications, including the above-mentioned cases with ascending upper-limb paresis, myeloradiculitis, and polyradiculoneuritis. We conducted the present study to retrospectively analyze these 15 patients' cases regarding host factors, HZ skin rash, neurological findings, virological testing, CSF, and magnetic resonance imaging (MRI) findings.

2. Patients and Methods

From June 2014 to March 2021, 15 patients with HZ/VZV peripheral nerve complications and HZ segmental paresis were seen at our hospital's departments of neurology and cerebrovascular medicine: segmental upper-limb paresis (n = 5), ascending upper-limb paresis (n = 2), ascending myeloradiculitis (n = 1), PHN (n = 1), polyradiculoneuritis (n = 3), and Elsberg syndrome (n = 3). Eleven of the patients required hospitalization due to limb paresis or four-limb paresis with radicular pain, as well as urinary retention and fecal incontinence for 1–3 months. We used the patients' hospitalization histories and outpatient records for the present analyses.

We focused on the HZ peripheral nerve complications unrelated to the dermatome concerning host risk factors, HZ skin rash, immunologic test results, neurological findings, anti-ganglioside (GM)1 antibody, VZV antibody, the VZV polymerase chain reaction (PCR) result for the CSF, a nerve conduction velocity including the F-wave, and MRI findings. The MRI examinations provided contrast-enhanced T1-weighted fat-suppressed images in 11 cases, and 3D NerveVIEW (Philips, Best, the Netherlands) MRI was performed in four patients at the acute stage for the cervical spine, thoracic spine, and lumbar spine.

This study was conducted in accord with the principles of the Declaration of Helsinki and was approved by our Hospital's Ethics Committee (Ron 21–203). Each of the patients provided informed and written consent to have their anonymized data and images published.

3. Results and Discussion

The results of the 15 Japanese patients with HZ peripheral nerve complications are summarized in Table 1, and the

Table 1. Fifteen patients with herpes zoster peripheral nerve complications (June 2014 to March 2021)

Pt. no.	Age, Sex	HZ dermatome, Radicular pain -, +, ++ Underlying disease	Paresis [a]after or [b]before HZ Adm	CSF cells /μL CF	Spinal MRI including 3D NerveVIEW	NCV study F-wave
1	63, F	HZ duplex, R VIII, L-C, ++, None	R-Hunt, L-upper-limb paresis, [a]5 days, Adm	21 cells 256x	High-intensity lesions at C5-8 nerve root	Upper-limb NCV & F-wave NWL
2	62, M	L-C6-Th1, ++ CS	L-upper-limb paresis [a]5 days	ND 256x	High signals with enhancements at C5-8	Needle EMG; Fib+ F-wave WNL
3	78, M	Disseminated, ++ CS, Renal disease	R-upper-limb paresis [a]5 days, Adm	ND 128x	Not detected	Needle EMG Fib+ F-wave decreased
4	77, F	R-C8-Th1, ++ CS	R-upper-limb paresis [a]5 days, Adm	4 cells 256x	Not detected	Upper-limb NCV & F-wave WNL
5	79, F	R-6-7, + CS	R-upper-limb paresis [a]2–3 days	ND 128x	Cervical MRI not detected	Upper-limb NCV WNL
6	64, M	L-Th8, ++ None	PHN, [a]1–2 weeks	ND 32x	3D Nerve VIEW High-intensity at Th8	ND
7	53, F	L-Th4, + Breast cancer	L-upper-limb paresis [a]4–5 days, Adm	ND 32x	High-intensity lesions at C8-Th4 nerve roots	ND
8	72, F	R-Th7-8, + None	R-upper-limb paresis [a]1 month	ND	Thoracic MRI not detected	MCV SCV WNL
9	32, F	R-Th11-12, + None	Ascending myelo- radiculitis, [a]40 days, Adm	19 cells 64x	MRI T1WI high signals at Th2-Th8,	ND
10	76, F	R-C7-Th1, + Interstitial pneumonia	Polyradiculoneuritis,[b]2–3 days	21 cells IgM+	MRI T1WI high signals at C5-8, L4-S2	R-dominant demyelinating
11	34, M	Disseminated, ++ Stress	Polyradiculoneuritis Same time, Adm	32 cells 128x	3D NerveVIEW high signals at C4-T1, L2-5	Lower-limb F-wave decreased
12	82, F	L-Hunt, − None	Polyradiculoneuritis, [b]2–3 days, Adm	143 cells PCR+	3D NerveVIEW high signals at L2-5, S1-2	Lower-limb F-wave decreased
13	62, M	L-S2-3, − None	Elsberg syndrome Same time, Adm	156 cells PCR+,32x	Contrast MRI T1WI not detected	ND
14	67, F	R-S-2-3, −	Elsberg syndrome [b]4 days, Adm	ND 512x	Contrast MRI T1WI High signals at R-S 2-3	ND
15	74, F	R-S3-5, + Colon cancer	Elsberg syndrome [a]5–6 days, Adm	11 cells	Contrast MRI T1WI not detected	ND

Adm: admission, C: cervical, CF: complement fixation titer, CS: cervical spondylosis, CSF: cerebrospinal fluid, F: female, Fib: fibrillation potential, HZ: herpes zoster, L: left, M: male, NCV: nerve conduction study, ND: not down, PCR: polymerase chain reaction, Pt: patient, R: right, S: sacral, Th: thoracic, WNL: within the normal limit.

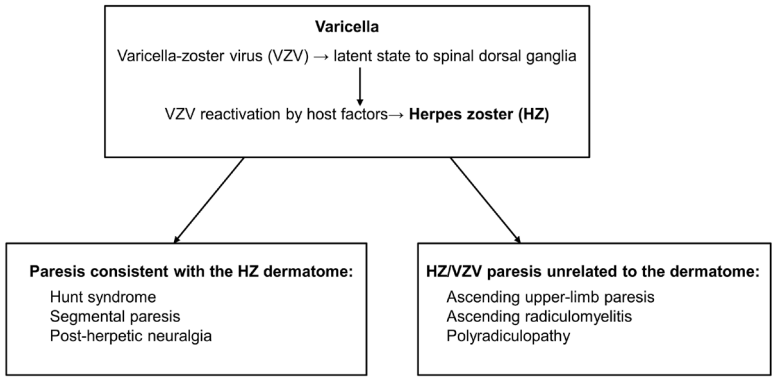

Figure 1. Herpes zoster (HZ) peripheral nerve complications. These usually appear at the segmental regions, consistent with the dermatome, but in our present patient series, there were six cases of rare upper-limb paresis, myeloradiculitis, or polyradiculoneuritis that differed from the patient's HZ dermatome.

Table 2. Clinical characteristics of patients with herpes zoster peripheral nerve complications (n = 15, 2014–2021)

Clinical characteristics	Patients
Underlying disease:	
Malignancy	3 (20.0%)
Cervical spondylosis	3 (20.0%)
Interstitial pneumonia	1 (6.7%)
Stress	1 (6.7%)
None	7 (46.7%)
Herpes zoster rash:	
Disseminated rash	2 (13.3%)
Cranial nerve VIII region	2 (13.3%)
Cervical region	4 (26.7%)
Thoracic region	4 (26.7%)
Sacral region	3 (20.0%)
HZ peripheral paresis:	
Segmental upper-limb paresis	5 (33.3%)
Ascending upper-limb paresis	2 (13.3%)
Post-herpetic neuralgia	1 (6.7%)
Ascending myeloradiculitis	1 (6.7%)
Polyradiculoneuritis	3 (20.0%)
Elsberg syndrome	3 (20.0%)
Radicular pain:	
–	3 (20.0%)
+	6 (40.0%)
++	6 (40.0%)
Paresis after or before HZ rash:	
After	10 (66.7%)
Same time	2 (13.3%)
Before	3 (20.0%)

The data are for five males and 10 females, age 65 ± 0 yrs (mean ± SD).

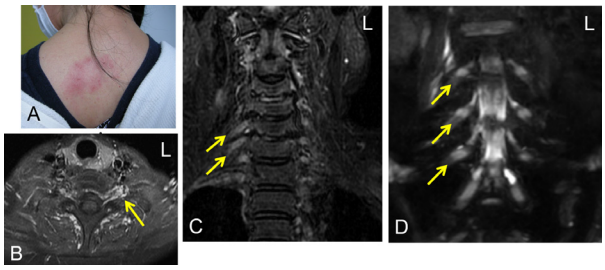

Figure 2. Herpes zoster (HZ) skin rash and MRI findings. (A) Patient 7 developed HZ in the left back of her trunk on the Th4 dermatome, and 5 days later she showed upper-limb paresis in the left C8 region. **(B)** Contrast MRI T1WI revealed high-intensity lesions with enhancements at the C8 nerve root region (*arrow*) (*7*). **(C)** Patient 10: Gd-enhanced cervical root coronary MRI T1WI showed high signals on both sides at the C6 and C7 nerve roots (*right side arrows*) (*10*). **(D)** Patient 12: Lumbar root coronary MRI using 3D NerveVIEW imaging showed high-intensity lesions on both sides at L2–4 spinal roots (*arrows*) (*12*). The HZ skin rash and CT/MRI images are reprinted with permission from references (*7*), (*10*), and (*12*).

patients' clinical characteristics are described in Table 2. The average age of the 15 patients (five males, 10 females) was 65 years. Eleven patients required 2–3 months' hospitalization at the acute stage for antiviral therapy, severe pain, and urinary impairment. We found no history of VZV vaccination among the patients.

Eight patients had underlying diseases: malignancy (n = 3), cervical spondylosis (n = 3), interstitial pneumonia (n = 1) and stress (n = 1), and the other seven patients had no disease. Regarding the HZ dermatome, we identified generalized HZ rash (n = 2), cranial nerve region (n = 2), cervical region (n = 4), thoracic region (n = 4), and sacral region (n = 3). Concerning radicular pain, the medical records revealed patients without pain (n = 2), mild-moderate + pain (n = 6), and severe ++ pain (n = 6). GM1 antibody was negative in Patients 10 and 12. The serum complement fixation (CF) antibody values for VZV were 32x–512x (positive < 8x in 11 patients), in which CF antibodies were parallel with the enzyme-immunosorbent assay IgM value (data not shown). In serum and CSF, the VZV PCR result was positive in Patients 12 and 13. An increase in the CSF cell number > 5 cells was observed in seven of the eight patients examined (mean 50.9 cells, range 4–518/μL).

HZ peripheral nerve complications are usually segmental paresis consistent with the dermatome (*13,14*). However, in the present patient series, there were six cases of rare upper-limb paresis (n = 2), myeloradiculitis (n = 1), and polyradiculoneuritis (n = 3) that differed from the HZ dermatome (Figure 1). Patient 7 presented segmental upper-limb paresis in the left C-8 region at 5 days after an HZ rash had developed on the Th4 dermatome, and contrast MRI T1WI showed high signals with enhancements at C8 nerve root regions; ascension along spinal roots was suspected to be the VZV propagation route (Figure 2, A and B) (*7*).

Patient 9, a 32-year-old woman, developed ascending myeloradiculitis after exhibiting an HZ rash at the Th11–12 dermatomes, and the demyelinating process after HZ infection was suspected due to the patient's high myelin-

basic protein titer (*8*); the peak value was 942 pg/mL at the acute stage and then decreased. Patient 1, an elderly woman who had an HZ duplex in her right auricle and left shoulder, developed right Hunt syndrome, followed by left upper-limb paresis (*9*). Our search of the literature indicated that HZ segmental limb-paresis distant from the dermatome or HZ demyelinating myeloradiculitis is relatively rare (*15,16*).

Cortese *et al.* reported the case of a 79-year-old man with flaccid paralysis of the lower limbs without HZ rash; CSF pleocytosis and a VZV DNA PCR-positive result were detected in his CSF, and MRI findings showed cauda equina radiculopathy (*17*). We later reported the cases of the following three patients with VZV polyradiculoneuritis that differed from their HZ dermatomes (*10-12*). Patient 10, an elderly woman, presented VZV-associated polyradiculoneuritis, ascending motor paralysis in the limbs, and a CSF cell increase (21 cells/μL); VZV IgM antibody persisted, and cervical and lumbar spine MRI contrast T1WI revealed high-intensity lesions in the C7–Th1 (Figure 2C) (*10*) and L4–5 spinal root regions. Patient 11 exhibited quadriplegia in the proximal lower limbs with disseminated HZ (*11*). Patient 12 showed VZV polyradiculoneuritis that had initially developed in the lower spinal ganglia, in which 3D NerveVIEW and MRI T1WI showed high signal intensities at the L2–4 spinal ganglia and roots (Figure 2D) (*12*).

These patients' cases shared the characteristic of main lesions in the spinal nerve root, and their cervical and lumbar MRI examinations using 3D NerveVIEW imaging clearly revealed lumbar ganglia and nerve root lesions. These MRI findings may suggest VZV propagating routes. We speculate that our three above-described patients may have had a new subtype of HZ peripheral neuropathies that differs from that of GBS. Based on the clinical findings and MRI results of the patients' spinal ganglia and roots, it appears that the patterns of VZV spread follow three or four routes: *i*) ascending spinal roots, *ii*) ascending spinal cord, *iii*) polyradiculopathy, and *iv*) intrathecal spread. The patients' polyradiculopathy originating from spinal ganglia or ascending radiculomyelitis including neuropathies varied, but in our comparison of the group of five patients with paresis consistent with the dermatome, no differences were identified in terms of host factors, underlying disease, CF antibody titer, or CSF cells.

Regarding the incidence of Elsberg syndrome showing urinary dysfunction and meningitis, Patients 13–15 had Elsberg syndrome involving sacral-region HZ with urinary injury and meningitis (*18,19*). Patient 14 presented with urinary and fecal incontinence, and MRI on the cauda equina nerve showed high-intensity lesions.

HZ/VZV neurological infections are treated with specific antiviral drugs, steroid hormones, and/or high-dose intravenous immunoglobulin therapy (IVIg), but sequelae such as PHN persist for a long time, significantly impairing patients' activities of daily living and quality of life. It is expected that the generalization of live varicella vaccines or subunit vaccines for the elderly will lead to a reduction in the risk of HZ infection (*20*).

The limitations of this study are as follows. Its design was a retrospective clinical analysis. CSF testing and spinal MRI including 3D NerveVIEW had not been performed for all patients. There were also few cases of HZ peripheral nerve complications associated with an unrelated dermatome, and no autopsies to support the pathology of spinal ganglia and roots in HZ/VZV peripheral nerve complications were available.

In conclusion, we analyzed host factors, skin lesions, and the neurological, virological and MRI/3D NerveVIEW findings in 15 patients with HZ peripheral nerve complications. Six of the patients had the rare conditions of upper-limb paresis, myeloradiculitis, or polyradiculoneuritis that differed from the HZ dermatome. Based on the clinical findings and MRI results of the patients' spinal ganglia and roots, we speculate that the patterns of VZV spread involve ascending spinal roots or ascending spinal cord, polyradiculopathy, and intrathecal spread. The VZV propagation process from VZV reactivation varied among the present patients. The incidence of HZ is increasing with the aging of many populations, and clinicians should thus be aware of the potential HZ neuropathies described herein.

Funding: This study was supported by a St. Mary's Hospital Research Fund (no. Ron 23-0907).

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

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Received October 7, 2023; Revised November 8, 2023; Accepted November 14, 2023.

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Released online in J-STAGE as advance publication November 18, 2023.

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DOI: 10.5582/irdr.2023.01086

Release and impact of China's "Second List of Rare Diseases"

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SUMMARY On September 18, 2023, the National Health Commission of China officially announced the "Second List of Rare Diseases". This list of 86 rare diseases, drafted in accordance with the "Working Procedures for Drafting the List of Rare Diseases", marks the second release of a rare disease list since the initial list was issued in May 2018. Following the release of the first batch, the Chinese Government introduced various policies to enhance the diagnosis and treatment of rare diseases, to promote the research on, development of, production of, and availability of rare disease medications in China, and to improve medication access for patients with rare diseases. Consequently, this has elevated the level of rare disease diagnosis and treatment, ensuring greater accessibility to treatment for affected individuals. The expansion of the rare disease list through the release of the "Second List of Rare Diseases" will further enhance rare disease management, increase awareness, improve diagnosis and treatment, facilitate the development and availability of more rare disease medications, establish a comprehensive support system for patients with rare diseases, and ultimately benefit a larger number of individuals affected by rare diseases. The definition of rare diseases in China should be refined by explicitly establishing corresponding criteria based on incidence, prevalence, or the number of affected individuals. Additionally, the mechanism for removal of diseases from rare disease lists should be enhanced, and prompt adjustments should be made regarding diseases that do not align with the selection principles of the list, taking into consideration environmental changes.

Keywords rare diseases, China, disease list, health policy, definition

1. Introduction

On September 18, 2023, six departments in China, including the National Health Commission, the Ministry of Science and Technology, the Ministry of Industry and Information Technology, the National Medical Products Administration, the National Administration of Traditional Chinese Medicine, and the Logistics Support Department of the Central Military Commission, jointly released the "Second List of Rare Diseases". This list includes a total of 86 rare diseases (*1*), such as acromegaly, narcolepsy, beta-thalassemia major, neuroblastoma, and clear cell sarcoma of the kidney. It covers 17 medical specialties, primarily including hematology, dermatology, rheumatology and immunology, pediatrics, neurology, and endocrinology. Combined with the 121 rare diseases listed in the "First List of Rare Diseases" released in May 2018, the current list of rare diseases in China now has 207 rare diseases (*2*) (Tables 1 and 2). In comparison to the "First List of Rare Diseases", the diseases included in the "Second List of Rare Diseases" are associated with either marketed or investigational drugs, thus emphasizing the significant principle of "treatable with medicine". Moreover, a point worth noting is that, similar to the first list, the National Healthcare Security Administration is still not identified as the issuing body in the second list. Internationally, rare diseases are typically defined based on their prevalence or the number of affected individuals. China's efforts in rare disease-related work have been relatively delayed, and there is a scarcity of epidemiological data on rare diseases. Given this context, the approach of recognizing and managing rare diseases through a list aligns with the country's specific circumstances.

2. Procedures for the drafting of the "Second List of Rare Diseases"

The drafting of the "Second List of Rare Diseases" takes into account national conditions in China, including economic development, population dynamics, and social security levels. It also draws upon the management

Table 1. "First List of Rare Diseases" in China (2018)

No.	Diseases	No.	Diseases
1	21-Hydroxyulase deficiency	61	Laron syndrome
2	Albinism	62	Leber hereditary optic neuropathy
3	Alport syndrome	63	Long chain 3-hydroxyacyl-CoA dehydrogenase deficiency
4	Amyotrophic lateral sclerosis	64	Lymphangioleiomyomatosis (LAM)
5	Angelman syndrome	65	Lysine urinary protein intolerance
6	Arginase deficiency	66	Lysosomal acid lipase deficiency
7	Asphyxiating thoracic dystrophy (Jeune syndrome)	67	Maple syrup urine disease
8	Atypical hemolytic uremic syndrome	68	Marfan syndrome
9	Autoimmune encephalitis	69	McCune-Albright syndrome
10	Autoimmune hypophysitis	70	Medium chain Acyl-CoA dehydrogenase deficiency
11	Autoimmune insulin receptopathy (type B insulin resistance)	71	Methylmalonic acidemia
12	Beta-ketothiolase deficiency	72	Mitochondrial encephalomyopathy
13	Biotinidase deficiency	73	Mucopolysaccharidosis
14	Cardiac ion channelopathies	74	Multi-focal motor neuropathy
15	Carnitine deficiency	75	Multiple Acyl-CoA dehydrogenase deficiency
16	Castleman disease	76	Multiple sclerosis
17	Charcot-Marie-Tooth disease	77	Multiple system atrophy
18	Citrullinemia	78	Myotonic dystrophy
19	Congenital adrenal hypoplasia	79	NAGS deficiency
20	Congenital hyperinsulinemic hypoglycemia	80	Neonatal diabetes mellitus
21	Congenital myasthenic syndrome	81	Neuromyelitis optica
22	Congenital myotonia syndrome (non-dystrophic myotonia, NDM)	82	Niemann-Pick disease
23	Congenital scoliosis	83	Non-syndromic deafness
24	Coronary artery ectasia	84	Noonan syndrome
25	Diamond-Blackfan anemia	85	Ornithine transcarbamylase deficiency
26	Erdheim-Chester disease	86	Osteogenesis imperfecta (brittle bone disease)
27	Fabry disease	87	Parkinson disease (young-onset, early-onset)
28	Familial Mediterranean fever	88	Paroxysmal nocturnal hemoglobinuria
29	Fanconi anemia	89	Peutz-Jeghers syndrome
30	Galactosemia	90	Phenylketonuria
31	Gaucher's disease	91	POEMS syndrome
32	General myasthenia gravis	92	Porphyria
33	Gitelman syndrome	93	Prader-Willi syndrome
34	Glutaric acidemia type I	94	Primary combined immune deficiency
35	Glycogen storage disease (type I, II)	95	Primary hereditary dystonia
36	Hemophilia	96	Primary light chain amyloidosis
37	Hepatolenticular degeneration (Wilson disease)	97	Progressive familial intrahepatic cholestasis
38	Hereditary angioedema (HAE)	98	Progressive muscular dystrophies
39	Hereditary epidermolysis bullosa	99	Propionic acidemia
40	Hereditary fructose intolerance	100	Pulmonary alveolar proteinosis
41	Hereditary hypomagnesemia	101	Pulmonary cystic fibrosis
42	Hereditary multi-infarct dementia (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy, CADASIL)	102	Retinitis pigmentosa
		103	Retinoblastoma
		104	Severe congenital neutropenia
43	Hereditary spastic paraplegia	105	Severe myoclonic epilepsy in infancy (Dravet syndrome)
44	Holocarboxylase synthetase deficiency	106	Sickle cell disease
45	Homocysteinemia	107	Silver-Russell syndrome
46	Homozygous hypercholesterolemia	108	Sitosterolemia
47	Huntington disease	109	Spinal and bulbar muscular atrophy (Kennedy disease)
48	Hyperornithinaemia-hyperammoniaemia-homocitrullinuria syndrome	110	Spinal muscular atrophy
49	Hyperphenylalaninemia	111	Spinocerebellar ataxia
50	Hypophosphatasia	112	Systemic sclerosis
51	Hypophosphatemia rickets	113	Tetrahydrobiopterin deficiency
52	Idiopathic cardiomyopathy	114	Tuberous sclerosis complex
53	Idiopathic hypogonadotropic hypogonadism	115	Tyrosinemia
54	Idiopathic pulmonary arterial hypertension	116	Very long chain Acyl-CoA dehydrogenase deficiency
55	Idiopathic pulmonary fibrosis	117	Williams syndrome
56	IgG4-related disease	118	Wiskott-Aldrich syndrome
57	Inborn errors of bile acid synthesis	119	X-linked agammaglobulinemia
58	Isovaleric acidemia	120	X-linked adrenoleukodystrophy
59	Kallmann syndrome	121	X-linked lymphoproliferative disease
60	Langerhans cell histiocytosis		

experience of countries or regions with similar levels of social development. Spearheaded by the National Health Commission of China, the drafting of the List follows the guidelines outlined in the "Working Procedures for Drafting the List of Rare Diseases" issued by the National Health Commission in 2018. The National Health Commission oversees the Expert Committee on Diagnosis and Treatment of and Ensured Care for Rare Diseases (hereinafter referred to as the Expert Committee) and its corresponding office. The Expert Committee provides technical support and policy recommendations, while the office is responsible for day-to-day tasks such as receiving, collating, and organizing application materials for rare diseases.

Table 2. "Second List of Rare Diseases" in China (2023)

No.	Diseases	No.	Diseases
1	Achondroplasia	44	Lennox-Gastaut syndrome
2	Acquired hemophilia	45	Limbal stem cell deficiency
3	Acromegaly	46	Malignant hyperthermia
4	Adult-onset Still disease	47	Malignant pleural mesothelioma
5	Alagille syndrome	48	Melanoma
6	Alpha-1-antitrypsin deficiency	49	Metachromatic leukodystrophy
7	ANCA-associated vasculitis	50	Monogenic non-syndromic obesity
8	Bardet-Biedl syndrome	51	Multiple endocrine neoplasia
9	Behçet's disease	52	Narcolepsy
10	Blue rubber bleb nevus	53	Neuroblastoma
11	CDKL5 deficiency disorder	54	Neurofibromatosis
12	Choroideremia	55	Neuronal ceroid lipofuscinosis
13	Chronic inflammatory demyelinating polyneuropathy	56	Neurotrophic keratitis
14	Clear cell sarcoma of kidney	57	Osteosarcoma
15	Cold agglutinin disease	58	Pemphigus
16	Congenital biliary atresia	59	Persistent pulmonary hypertension of the newborn
17	Congenital factor VII deficiency	60	Pheochromocytoma
18	Cryopyrin-associated periodic syndrome/NLRP3-associated systemic autoinflammatory disease	61	PIK3CA-related overgrowth syndrome
		62	Polycythaemia vera
19	Cutaneous neuroendocrine carcinoma (Merkel cell carcinoma)	63	Primary biliary cholangitis
20	Cutaneous T-cell lymphomas	64	Primary growth hormone deficiency
21	Cystinosis	65	Primary IGF1 deficiency
22	Dermatofibrosarcoma protuberans	66	Primary immunodeficiency
23	Eosinophilic gastroenteritis	67	Primary myelofibrosis
24	Epithelioid sarcoma	68	Primary sclerosing cholangitis
25	Facioscapulohumeral muscular dystrophy	69	Progressive fibrosing interstitial lung disease
26	Familial hemophagocytic lymphohistiocytosis	70	Recurrent pericarditis
27	Familial adenomatous polyposis	71	Retinopathy of prematurity
28	Fibrodysplasia ossificans progressiva	72	Rett syndrome
29	Fragile X syndrome	73	Short bowel syndrome
30	Gangliosidosis	74	Systemic juvenile idiopathic arthritis
31	Gastroenteropancreatic neuroendocrine neoplasm	75	Systemic mastocytosis
32	Gastrointestinal stromal tumor	76	Takayasu arteritis
33	Generalized pustular psoriasis	77	Tenosynovial giant cell tumor/Pigmented villonodular synovitis
34	Genetic hypoparathyroidism	78	Thalassemia major
35	Giant cell arteritis	79	Thrombotic thrombocytopenic purpura
36	Giant cell tumor of bone	80	Transthyretin amyloidosis
37	Glanzmann thrombasthenia	81	Tumor necrosis factor receptor associated periodic syndrome
38	Glioblastoma	82	Tumor-induced osteomalacia
39	Gorlin syndrome	83	Von Hippel-Lindau syndrome
40	Hidradenitis suppurativa	84	Von Willebrand disease type3
41	Hutchinson-Gilford progeria syndrome	85	Waldenström macroglobulinemia/ Lymphoplasmacytic lymphoma
42	Inflammatory myofibroblastic tumor	86	West syndrome/Infantile spasms syndrome
43	Leber congenital amaurosis		

The application process for adding categories encompasses six stages: preparation of application materials, submission of materials, verification of information, research and demonstrations, solicitation of opinions, and final determination and publication of the list (see Figure 1 for a detailed overview). Applications to include disease categories in the list are made by provincial health administrative departments, national industry associations or societies, and registered civil organizations under the Ministry of Civil Affairs. The selection principle is based on evidence from both domestic and international sources, indicating a low incidence or prevalence, significant harm to patients and their families, clear methods of diagnosis, affordability of treatment or intervention measures, or the absence of an effective treatment or intervention measures, but inclusion in national research projects. Following deliberations by the Expert Committee, a preliminary list is drafted and widely circulated for public input. After considering the feedback, the final list is determined and subsequently issued by the National Health Commission (*3*). In accordance with the requirements outlined in the "Working Procedures for Drafting the List of Rare Diseases" the update cycle for the "List of Rare Diseases" in China is generally set at a minimum of two years. Five years have passed since the release of the first list of rare diseases in China, and the second batch is now being issued.

3. The impact of the list of rare diseases

The publication of the "First List of Rare Diseases" has provided a crucial reference for relevant departments to undertake rare disease-related work. With the release of this list, numerous complementary policies have been introduced by the National Health Commission, the National Medical Products Administration, the National Medical Insurance Administration, and other pertinent agencies. These policies aim to enhance the diagnostic and treatment of rare diseases in China, safeguard the

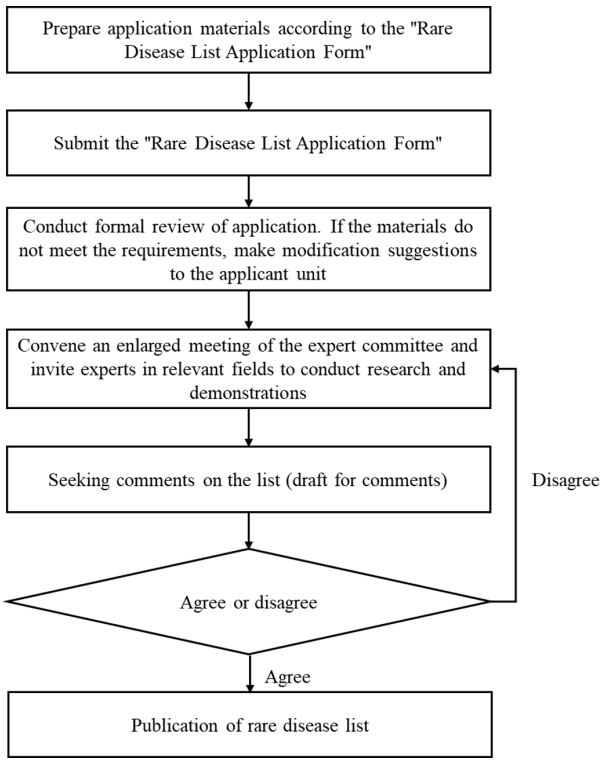

Figure 1. Application process for adding rare disease categories to the list

rights and interests of patients, improve the accessibility of treatment options, and foster the comprehensive development of the rare disease field.

3.1. Driving the enhancement of rare disease diagnosis and treatment capacity in China

In terms of promoting the improvement of diagnosis and treatment capabilities for rare diseases in China, in 2019, the release of the "First List of Rare Diseases" in China led to the release of the "Rare Disease Diagnosis and Treatment Guidelines (2019 Edition)", providing a solid foundation for rare disease diagnosis and treatment (*4*). In February 2019, the National Health Commission issued a notice to establish a national rare disease diagnosis and treatment collaboration network. This initiative involved the selection of 324 hospitals (1 national leading hospital, 32 provincial leading hospitals, and 291 member hospitals in the collaborative network) across the country with strong capabilities in rare disease diagnosis and treatment and a substantial number of cases, forming a collaborative network (*5*). The primary objective of this network is to provide concentrated diagnosis and treatment for patients with rare diseases and facilitate two-way referrals. The aim is to enhance the comprehensive diagnosis and treatment capabilities for rare diseases in China using quality medical resources. In October 2019, Peking Union Medical College Hospital was entrusted by the National Health Commission to develop the "China Rare Disease Diagnosis and Treatment Service Information System". This system requires hospitals in the collaborative network to promptly report information on patients they see with rare diseases from the first list of rare diseases (*6*). By collecting data on the diagnosis, distribution, and other relevant information regarding rare diseases in China, this system aims to provide scientific evidence for the formulation of population intervention strategies, improvement of the diagnosis and treatment system, and enhancement of drug accessibility. In February 2020, The National Rare Disease Diagnosis and Treatment Collaboration Network Office was officially established at Peking Union Medical College Hospital in Beijing (*7*). Additionally, in November 2020, the National Health Commission entrusted Peking Union Medical College Hospital to establish a national-level rare disease quality control center. The center focuses on implementing standardized diagnosis and treatment quality control measures for rare diseases, with the goal of improving the level of rare disease care. The implementation of a series of policies has resulted in continuous improvements in the level and standardization of rare disease diagnosis and treatment in China. Nevertheless, the rare disease diagnosis and treatment system in China is still in its early stages.

3.2. Encouraging the development and market approval of rare disease drugs in China

In terms of encouraging the research, production, and market launch of rare disease drugs in China, promoting the development and availability of drugs for rare diseases in China has been a priority for various government departments, including the National Medical Products Administration, the Ministry of Science and Technology, and the Ministry of Finance. These departments have implemented measures to expedite the evaluation and approval of rare disease drugs, enhance research and development efficiency, increase funding for research, and provide tax incentives (*8*). In October 2018, the National Medical Products Administration and the National Health Commission jointly established a special pathway for the evaluation and approval of urgently needed new drugs from overseas. This pathway focuses on new drugs for the treatment of rare diseases that have been approved in the United States, the European Union, or Japan but are not yet available in China, as well as drugs for severe life-threatening or life-impairing diseases without effective treatment options or with significant clinical advantages. A total of 40 rare disease drugs have been included in the three batches of lists issued through this pathway, and 26 of them have been approved for market (*9*). In January 2020, the revised "Drug Registration Management Measures" introduced a priority evaluation and approval process for innovative drugs and improved new drugs for the prevention and treatment of rare diseases with clear clinical value (*10*).

The evaluation and approval time limit for rare disease drugs within this priority scope is 130 days, with a shorter time limit of 70 days for urgently needed rare disease drugs that are already available overseas but not yet in China. This has expedited the evaluation process. In December 2020, the National Medical Products Administration issued the "Management Measures for Communication and Exchange during Drug Research and Technical Evaluation", which established a communication mechanism to improve research and development efficiency and accelerate the progress of rare disease drug development and availability. In May 2022, the "Regulations Implementing the Drug Administration Law of the People's Republic of China (Draft for Solicitation of Comments)" proposed a policy recommendation of a maximum market exclusivity period of 7 years to support the research and development of rare disease drugs. Additionally, the Ministry of Science and Technology has provided financial support through national research programs to advance rare disease research and development, with an investment of approximately 120 million yuan from the central government. The notice on the value-added tax policy for rare disease drugs, jointly issued by the National Medical Products Administration, the Ministry of Finance, the General Administration of Customs, and the State Taxation Administration, provides tax exemptions for these drugs (*11*). Over the past two years, the National Medical Products Administration has issued "Guiding Principles for Clinical Development of Orphan Drugs" and "Guiding Principles for Disease Natural History Study in the Development of Orphan Drugs" in order to better guide the research and development of orphan drugs. They have also sought opinions on "Guiding Principles for Statistical Guidance in Clinical Research of Orphan Drugs", "Guiding Principles for Clinical Trials of Gene Therapy Products for Rare Diseases", and "Guiding Principles for Non-clinical Research of Enzyme Replacement Therapy Drugs for Rare Diseases". As a result of these policy incentives, China has successfully launched 75 rare disease drugs (*12*) in accordance with the "First List of Rare Diseases", and at least 41 drugs have been launched domestically for the treatment of 29 rare diseases listed in the "Second List of Rare Diseases". These efforts have improved the accessibility of rare disease drugs for patients in China and addressed the challenges of accessing medication for patients with rare diseases.

3.3. Ensuring medical care for patients with rare diseases in China

In terms of ensuring medical care for patients with rare diseases, China has established a comprehensive medical insurance system that includes employee medical insurance, urban and rural resident medical insurance, supplementary enterprise medical insurance, and urban and rural medical assistance. The "First List of Rare Diseases" has already included over 50 out of the 75 rare disease drugs available in China, in the "National Basic Medical Insurance, Work-related Injury Insurance, and Maternity Insurance Drug List" (referred to as the "Medical Insurance Drug List") (*12*). Twenty-six drugs were included through a negotiation process, with an average price reduction of over 50%. This significantly lowered the prices of drugs for rare diseases, and especially some extremely expensive ones. It effectively alleviated the financial burden on patients, such as for the treatment of Fabry disease with agalsidase-α, spinal muscular atrophy (SMA) with nusinersen, and risdiplam. Moreover, the "Second List of Rare Diseases" has included three drugs in the medical insurance drug list, and multiple drugs have been submitted to the National Healthcare Security Administration for inclusion in the list. Additionally, the government has implemented a centralized procurement policy to include rare disease drugs, significantly reducing their prices. The drug Ambrisentan, used to treat idiopathic pulmonary arterial hypertension, has experienced a price drop from 115.97 yuan per tablet to 20 yuan per tablet through the national centralized procurement program. In provinces and cities where rare disease drugs are not covered by the medical insurance drug list, local models of coverage have been introduced, such as the rare disease drug special fund model in Zhejiang and Jiangsu provinces, and the "Hui Min Bao" commercial insurance model in many regions (*13*). China is also exploring the establishment of a multi-level insurance system, led by national medical insurance and shared with commercial insurance, charity, and medical assistance, to continuously improve the level of medical care for patients with rare diseases in the country.

4. Conclusion

The release of the "Second List of Rare Diseases" has expanded the scope of the rare disease list in China, providing a foundation for the implementation of future rare disease-related policies. This development will contribute to the strengthening of rare disease management in China, promoting greater awareness and enhancing the diagnosis and treatment of rare diseases. Additionally, it will facilitate the research on, development of, and availability of more rare disease drugs while establishing a robust system to safeguard the needs of patients with rare diseases and extend benefits to a larger population. An important point worth noting that while the procedures for drafting the list of rare diseases have outlined clear selection principles for rare diseases, the term "low incidence or prevalence" remains too ambiguous. The definition of rare diseases in China needs to be refined and specific criteria need to be established based on factors such as incidence, prevalence, or the number of affected individuals. The

definition of rare diseases and the list of rare diseases can coexist, with the former serving as a long-term strategic plan and the latter focusing on specific rare disease types and objectives within a defined timeframe. Additionally, a crucial step is to establish a mechanism for removing diseases from the list of rare diseases and promptly adjusting diseases that do not meet the list's selection principles based on changing circumstances.

Funding: This work was supported by a grant from the Young Talent Program from the Shanghai Health Commission (No. 2022YQ058).

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

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Received September 23, 2023; Revised October 4, 2023; Accepted October 8, 2023.

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Released online in J-STAGE as advance publication October 11, 2023.

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DOI: 10.5582/irdr.2023.01055

Vosoritide, a miracle drug, covering unmet need in achondroplasia: A regulatory update

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SUMMARY Dwarfism is a rare condition characterized by small stature. Achondroplasia is predominantly considered the leading cause of dwarfism. Although the condition is not life-threatening, it dramatically impacts the social life of the patient. The United States Food and Drug Administration (US FDA) first approved the drug Voxzogo (vosoritide) for achondroplasia. The drug also received approval from the European Medicines Agency (EMA) *via* the centralized procedure. The drug is associated with a decrease in blood pressure, a severe adverse event. However, this adverse event/risk has been overcome by benefits, *i.e.* fulfilling of unmet medical need. In the United States, the drug received accelerated approval as it satisfied the criteria of rare pediatric disease. This review includes a detailed orphan drug approval process with particular reference to vosoritide, which is considered a milestone for the treatment of achondroplasia.

Keywords US FDA's Orphan Products Grants Program, European Medicines Agency, vosoritide, achondroplasia, rare disease

1. Introduction

Rare diseases are diseases that affect only a small population. The definition of rare diseases varies from country to country based on their population. As per 10,000 people, a prevalence of less than 6.4 is considered rare disease in the United States (US); whereas a prevalence of less than 5 in European Union (EU) and Canada, and less than 4 in Japan and South Korea is regarded as the criteria for rare disease. Due to the lack of epidemiological data, prevalence-based definition has not been established yet in India (*1*). These rare diseases can be classified into two categories: life-threatening rare diseases and non-life-threatening rare diseases based on their severity. Tyrosinemia and lysosomal storage disorder are examples of life-threatening rare diseases. On the other hand, galactosemia and dwarfism are considered non-life-threatening diseases.

The drugs that are used in the treatment of rare diseases are called orphan drugs. Around 7,000–8,000 rare diseases have been identified so far and out of these only 5% have approved treatments. Disease specific treatment have been made available to a very marginal amount of patients, *i.e.*, less than 1 in 10 (*1*). There are multiple reasons for the unavailability of treatment for rare diseases. Among them, from the pharmaceutical company's perspective, the primary ones include less targeted population, more investment to study discrete physiological functions, less profit, and difficulty in conducting clinical trials in a small population. Whereas, from the regulatory bodies' perspective, separate guidelines for clinical trials related to rare diseases, less collaboration with pharmaceutical companies, and a weak framework of regulations regarding rare diseases are the major reasons. Apart from this, although treatment is available, it is not affordable by the patients.

Vosoritide is considered a milestone in treating dwarfism associated with achondroplasia: a genetic disorder in which mutation occurs in the fibroblast growth factor receptor 3 (FGFR3) gene responsible for converting cartilage into bones especially in the long bones of the arms and legs. In the US, fewer than 50,000 people are suffering from achondroplasia, making this a rare disease (*2*). Dwarfism has been recognized since ancient years, and evidence for the same can be found in the artworks of Rome, Greece & Egypt. The term achondroplasia was used in the first place about 100 years ago by Jules Parrot, a noteworthy figure in French pediatrics. However, previously dwarfism was not considered a disease, but now it has been included in the rare disease list of the United States (*3*). Achondroplasia is the primary and most common cause of dwarfism worldwide.

2. Achondroplasia

Achondroplasia is an autosomal-dominant disorder which is caused by change or mutation on the transmembrane portion of the FGFR-3 gene. In the mutation process, a glycine amino acid is replaced by arginine (G380R or Gly380Arg) at protein position 380 of the gene FGFR-3. Mutation at Gly380 leads to a 100% chance of achondroplasia, whereas mutation at Gly375 may lead to achondroplasia. The term achondroplasia literally implies the meaning: "without cartilage formation". Ossification, or the process of turning cartilage into bone, is the concern with achondroplasia, notably in the long bones of the arms and legs (*4*). A diagrammatic representation of the mutation sites for achondroplasia has been given in Figure 1.

Diagnosis of achondroplasia can be made through radiological and clinical feature analysis (*5*). Apart from the shortage of limbs, the disorder may also lead to other health conditions i.e, lordotic lumbar spine, apnea (breathing interruptions), ear infections, obesity, spinal stenosis and hydrocephalus (fluid build in the brain). Some studies also state that it impacts the lifespan of the patient.

In Japan, growth hormone is administered in the treatment of achondroplasia, which shows efficacy for up to 2 years, but many health complications are associated with it. An alternative to the former is a limb elongation procedure, which is also controversial (*6*). Vosoritide is a drug recently approved by both United States Food and Drug Administration (US FDA) and European Medicines Agency (EMA) for treating achondroplasia.

3. Vosoritide

Vosoritide resembles C-type natriuretic peptide (CNP) made up of 39 amino acids. Vosoritide binds to Natriuretic Peptide Receptor B (NPR-B), activates it and restores chondrogenesis, a process through which cartilage is developed. The chemical structure of vosoritide and C-type natriuretic peptide (CNP) is given in Figure 2. Activation of NPR-B leads to inhibition of the downstream signaling of the gene FGFR-3. Downstream signaling of the FGFR-3 gene inhibits proliferation and differentiation in bones leading to dwarfism. Vosoritide inhibits Rapidly Accelerated Fibrosarcoma Kinase-1 and by this promotes proliferation and differentiation within bones leading to the cure of dwarfism. The diagrammatic representation of the mechanism of vosoritide is given in Figure 3.

4. Regulatory approval aspects of the drug

Vosoritide received marketing authorization approval under the category of orphan drugs by EU and the US. Both the countries have different guidelines regarding the approval process of orphan drugs. In the EU, nearly 36 million people have been affected by more than 6,000 rare diseases. Of these, 80% are genetic, and around 70% develop in the pediatric stage. This accounts for the fact that 60% of orphan drugs are pediatric. According to EMA, a drug used in the treatment, prevention or diagnosis of a severe and life-threatening or chronically debilitating disease, affecting not more than 5 in 10,000 people is considered an orphan drug. The drug should also demonstrate significant benefit to any existing method of diagnosis, treatment or prevention of the condition (*7,8*). EMA assigns Committee for Orphan Medicinal Products (COMP) to examine whether the drug fulfills the criteria of being designated an orphan drug in the EU. The committee takes 90 days to evaluate the same.

The drug sponsor is eligible for a range of incentives and fee reductions which would encourage them to develop orphan drugs for rare diseases. EMA also offers scientific advice or protocol assistance, which aids the

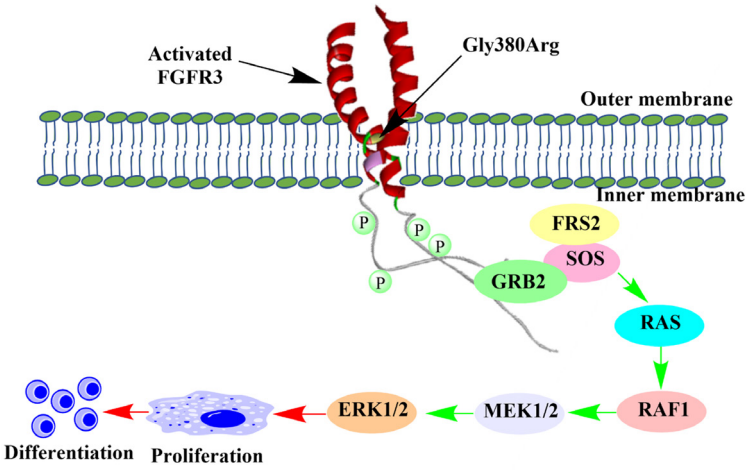

Figure 1. Mechanism of achondroplasia. Green arrow shows initiation; Red arrow shows inhibition. FGFR3: fibroblast growth factor receptor 3, Gly380Arg: mutated amino acid, GRB2: growth factor receptor-bound protein 2, FRS2: fibroblast growth factor receptor substrate 2, RAF1: raf-1 kinase, MEK1/2: mitogen-activated protein kinase kinases 1 and 2, ERK1/2: extracellular signal-related kinases 1 and 2, cGMP: cyclic guanidine monophosphate.

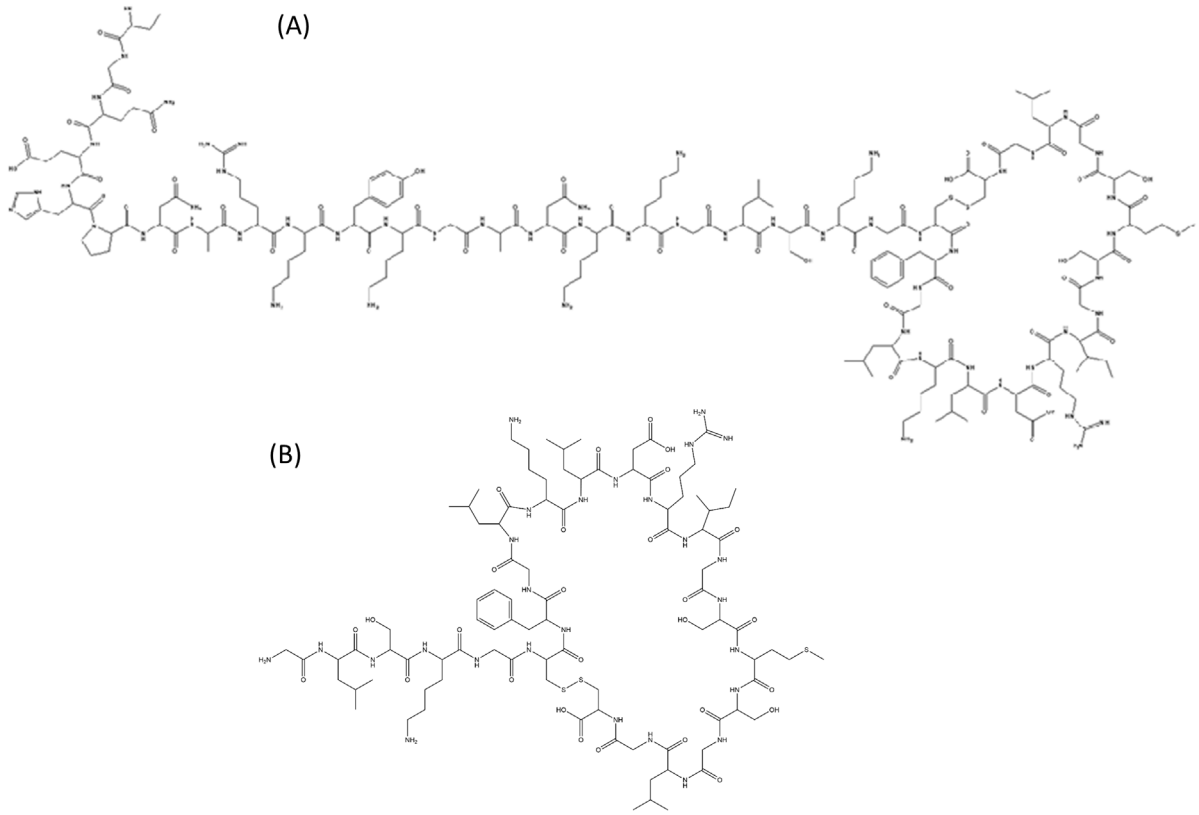

Figure 2. Chemical structure of (A) vosoritide and (B) C-type natriuretic peptide (CNP).

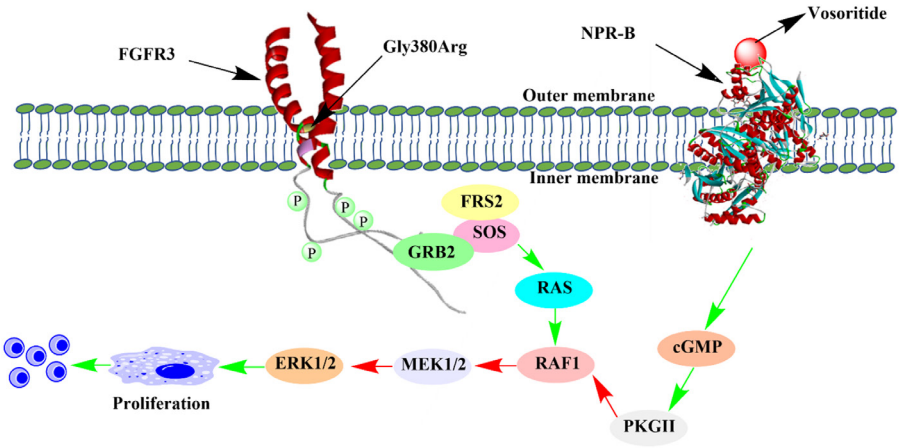

Figure 3. Mechanism of action of vosoritide on achondroplasia. Green arrow shows initiation; Red arrow shows inhibition. FGFR3: fibroblast growth factor receptor 3, Gly380Arg: mutated amino acid, GRB2: growth factor receptor-bound protein 2, FRS2: fibroblast growth factor receptor substrate 2, RAF1: raf-1 kinase, MEK1/2: mitogen-activated protein kinase kinases 1 and 2, ERK1/2: extracellular signal-related kinases 1 and 2, cGMP: cyclic guanidine monophosphate.

sponsor concerning quality, clinical, non-clinical aspects, and overall development strategy of the drug. In the case of micro, small and medium sized enterprises (SMEs), a fee reduction of up to 90% is applicable (*9*). The most attractive incentive offered by the European Commission to pharmaceutical companies is the 10-year market exclusivity period. This period can be extended by an additional two years if they are used in the pediatric population. Furthermore, orphan drugs have access to a centralized authorization procedure. They are also applicable for conditional approval under centralized procedures (*10*).

According to US FDA Orphan Drug Act, diseases affecting less than 200,000 people are regarded as rare diseases. In the US, 30 million people are affected by over 7,000 rare diseases. Before 1983, pharmaceutical

Table 1. Comparative table describing the orphan drug designation and incentives offered by EU and US

European Union (EU)	United States (US)
Prevalence calculation: Less than 5 in 10,000 in whole EU	Prevalence calculation: Less than 200,000 people in the US
10 years market exclusivity period (extended to 12 years if pediatric investigation plan fulfilled and implemented)	7 years market exclusivity period
EMA provided no funding.	Funding provided by US FDA
200 marketed orphan drug	100 approved orphan drug
Incentives applicable to only very serious and life-threatening, chronically debilitating disease	Incentives applicable to all kinds of rare diseases listed in the US
Pathways: Centralized marketing authorization and conditional approval	Pathways: Accelerated and prior approval
Waiver or fee reductions for marketing authorization application	Exemption from user fees for marketing application

companies had displayed no interest in developing orphan drugs as it was a very challenging task. This was because of the inherently low patient population, high cost of drug development, and the challenge in conducting clinical trials. In 1983, the US FDA developed the Orphan Drug Act which attracted drug developers as it provided incentives for orphan drug development. The sponsors can apply for various incentives such as a 7 year market exclusivity period after approval, tax credits for clinical testing, and Prescription Drug User Fee waiver (*11*).

Moreover, the US FDA also provides funding to the orphan drug developers through the Orphan Products Grants Program. This program has supported and funded clinical trials since 1983 and has assisted in approval of more than 80 products. The US FDA provides priority review vouchers for rare pediatric disease sponsors according to Section 529 of the Federal Food, Drug, & Cosmetic Act, which was added by the Food and Drug Administration Safety and Innovation Act (FDASIA). On 14th October 2021, the US FDA awarded 11 research grants for conducting clinical trials for rare diseases (*12*). Moreover, nowadays researchers are focusing on multiple unique approaches to develop treatment of rare diseases. In early 2023, in silico approach developed the first artificial intelligence treatment for idiopathic pulmonary fibrosis, which is a rare disease. This treatment reached up to phase II clinical trial in the US (*13*). The plant extract cannabidiol was also found to be effective in Dravet syndrome (rare epilepsy), which is also considered a rare disease. Achondroplasia is listed as a rare disease by the National Organization for Rare Disorders (NORD), which is an American non-profit organization. According to NORD, nearly 1 in 15,000 to 30,000 births are affected by achondroplasia in the US (*15*). On 19th November 2021, the US FDA approved the first treatment for Achondroplasia. The drug, Vosoritide has been approved through an accelerated approval pathway because the drug accomplished the unmet medical need of the disease (*16*). The application also went through prior approval, but final approval was sought through the accelerated approval pathway. Accelerated drug approval is provided based on the surrogate endpoint in clinical trials. For instance in cancer treatment, the sponsor cannot wait until evidence has been generated that the drug extends survival (clinical endpoint) in patients and so the agency can approve the drug based on the evidence that the treatment shrinks tumours (surrogate endpoint) (*17*).

A comparison between the orphan drug designation criteria and the incentives offered in the EU and the US has been given in Table 1. After comparing the regulations regarding orphan drugs in the two countries, it is observed that the EU has approved 200 drugs for rare diseases, which is double compared to the US. Although EMA does not provide funding directly and also considers only serious, life-threatening and chronically debilitating disease treatment under orphan designation, it still offers 10-year exclusivity for orphan drugs, which attracts many pharmaceutical companies towards it.

In August 2021, EU was the first country that approved vosoritide to treat achondroplasia in children aged ≥ 2 years with open epiphysis (*18*). The approval was given through a centralized procedure because the drug fulfilled all the requirements needed (*19*). 35 participants were enrolled in the 2nd phase of clinical trial, and during the 30-month cohort study, there were no severe adverse effects observed in participants. The trial was carried out in children aged 5 to 14 years (*20*).Moreover, in the 3rd phase of clinical trial, 119 participants entered into an extensive open-label study after placebo. No new adverse event was noticed in the 3rd clinical trial for a duration of up to 2 years (*21*).

5. Conclusions

Pharmaceutical companies are afraid to develop orphan drugs due to low targeted population and high investment. Moreover, the exclusive exemptions, fee waivers and patent exclusivity provided by the regulatory bodies encourage the companies to work on

orphan drugs. However, most of the researchers work on life-threatening rare diseases. Approval of vosoritide opens the path for the development of other non-life-threatening rare disease treatments. Approximately 6,000 rare diseases are in the EU and there are only 200 approved orphan drugs. Moreover, in the US there are only 100 approved orphan drugs. This data emphasized a huge gap in the disease and treatment in the case of rare diseases. More incentives and collaboration between the regulatory bodies and pharmaceutical companies are required to fill the gap.

Acknowledgements

We acknowledge the administrative support of National Institute of Pharmaceutical Education and Research (NIPER), Hyderabad.

Funding: None.

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

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Received July 10, 2023; Revised November 2, 2023; Accepted November 17, 2023.

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Released online in J-STAGE as advance publication November 19, 2023.

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DOI: 10.5582/irdr.2023.01060

Trofinetide in Rett syndrome: A brief review of safety and efficacy

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SUMMARY Rett syndrome (RTT) is a rare genetic neurological disorder that primarily affects girls and is caused by mainly mutations in the methyl-CpG-binding protein 2 (*MECP2*) gene, leading to critical issues in normal brain function. The condition has a global prevalence of 5 to 10 cases per 100,000 females, and there is currently no cure for RTT. However, therapy is available to manage the symptoms and improve quality of life. Trofinetide, an insulin-like growth factor 1, was originally developed as a stroke medication and progressed to Phase II clinical trials, where it exhibited favorable safety and efficacy profiles by improving several core RTT symptoms. Recently, Trofinetide received the US Food and Drug Administration (FDA) approval and orphan drug designation for the treatment of RTT, making it the first approved drug for this rare genetic disorder. It has also shown to be safe, well-tolerated and with no known drug interactions. These findings suggest that Trofinetide is a promising treatment option for individuals with RTT.

Keywords Rett syndrome, RTT, MECP2 gene, Trofinetide

1. Introduction

Rett syndrome (RTT) is a rare genetic neurological disorder that primarily affects girls and is mainly caused by mutations in the methyl-CpG-binding protein 2 (*MECP2*) gene. The *MECP2* gene leads to synthesis of a protein *i.e.*, MECP2, which is critical for normal brain function (*1*). *MECP2* gene has different functional domains *i.e.*, N-terminal domain (NTD), methyl binding domain (MBD), intervening domain (ID), transcription repression (TRD) and C-terminal. RTT develops due to various types of mutations *i.e*., missense, nonsense, frame shift, deletions and others. Structurally, mutations can involve NTD, MBD, other parts of gene. The mutations in different locations give rise to variable phenotypes with varying severity (*1*).

RTT starts developing between 6-18 months of age following apparently normal development in initial life. In the early onset or stagnation stage, the child may experience a slowing of head growth, delays in motor milestones, and loss of muscle tone (*2*). The rapid destructive stage is characterized by increased irritability, crying, decreased interest in social interaction, and repetitive hand movements (*2*). The plateau or pseudo-stationary stage may last for several years and is marked by stabilization of behavior and slight improvement in some skills. In the late motor deterioration stage, the child may experience a decline in motor function, including loss of mobility, scoliosis, and muscle weakness (*3*). Furthermore, many patients do not present with all the symptoms of RTT and termed as atypical or variant RTT. Important variants of RTT are Hanefeld, Zappella, and Rolando and each variant is characterized by specific phenotypic properties (*4*). Other common symptoms of RTT include seizures, sleep disturbances, digestive problems, and problems with heart and lung function. Around two-thirds of patients with RTT claimed to have associated epilepsy. The most common seizures encountered in RTT are complex partial and generalized tonic-clonic seizures (*4*). The patients present with early onset of seizures in Hanefeld variant which is characterized by normal *MECP2* gene expression. The concomitant presence of epilepsy makes the RTT worse. The electroencephalogram (EEG) is almost always abnormal in beyond age three in RTT, however abnormal EEG is not an indication for anti-seizure drugs *per se* (*5*).

The prevalence of RTT remains 5 to 10 cases per 100,000 females globally without regional variability (*6*). There is no cure for RTT, but treatment includes physical, occupational, and speech therapy, as well as behavioral therapy and medication to manage seizures and other symptoms (*7*). Currently multimodal treatment approaches for management of RTT are symptomatic

and supportive. It includes physical therapy to maintain mobility, balance, weight-bearing in scoliosis. Occupational therapy aims to improve/maintain adequate use of hands, reduce stereotypic movements and help in day to day activities. Furthermore, speech and language therapy help to maintain social interactions. The patients also require feeding and physical assistance. The drugs are mainly useful in controlling seizures, constipation, and arrhythmias (*7*). Even before the identification of MECP gene mutations, several trials have been conducted. The opioid antagonist, naltrexone was assessed as a treatment for periodic breathing. Another study revealed that intravenous naloxone caused slowing of EEG. When the MECP mutations were identified, a study was conducted with folate-betaine as a potential treatment. Though the parent reported scoring was better with folate-betaine, no concrete evidence was made (*8*).

Research into potential treatments for RTT is ongoing, and scientists are exploring a variety of approaches, including gene therapy, stem cell therapy, and drugs that target specific aspects of the disease. Gene therapy involves delivering a functional copy of the *MECP2* gene to affected cells. Stem cell therapy aims to repair or replace damaged cells in the brain. Drugs that target specific aspects of the disease include compounds that increase MeCP2 protein levels or target pathways that are disrupted in RTT (*9*). Presently there is no definitive management of RTT hence, there is a dire need of drug(s) which can address the underlying genetic cause of the disorder.

Trofinetide which is an analog of insulin-like growth factor-1 (IGF1) was originally developed as a stroke medication and progressed to Phase II clinical trials, where it exhibited favorable safety and efficacy profile by showing improvement in several core RTT symptoms at its highest dose (*10*). Trofinetide, under the brand name Daybue™, has been given approval by the US Food and Drug Administration (FDA) as a treatment for RTT in patients aged two years and above (*11*).

2. Mechanism of action

Trofinetide, a synthetic peptide being explored as a potential treatment for RTT, is believed to have multiple effects on the brain, although its mechanism of action is not yet fully understood. One potential mechanism of action is the promotion of synaptic maturation and function. The development and function of synapses, including their ability to change in strength and number in response to experience, are critical roles played by IGF-1. Studies have revealed that Trofinetide can increase the expression of genes that contribute to synaptic function and enhance synaptic plasticity in animal models of RTT (*12,13*). This is significant as neuroinflammation is believed to play a role in the pathophysiology of RTT and other neurodevelopmental disorders. The drug exerts its effects by normalizing abnormal neuronal and glial function, resulting in anti-inflammatory and trophic effects. It achieves this by inhibiting astrogliosis and pathologic microglial activation, which are the primary contributors to brain inflammation and neuronal damage in RTT. Trofinetide also normalizes synaptic protein synthesis, dendritic morphology, and neuronal signaling, all of which are vital for proper neuronal function. Additionally, it enhances the antioxidant response, thus protecting neurons from oxidative stress-induced damage, which is a common feature of neurodegenerative disorders, including RTT (*14,15*). By reducing the levels of pro-inflammatory cytokines in the brain, Trofinetide helps reduce neuroinflammation, which may protect neurons from damage (*15*) (Figure 1).

3. Pharmacokinetics

Trofinetide is administered orally and the time taken to achieve maximum drug concentration (Tmax) is about ≅3 hours. The apparent volume of distribution and half-life of Trofinetide is 80L and ≅1.5 hours respectively. About 80% of the administered drug gets excreted

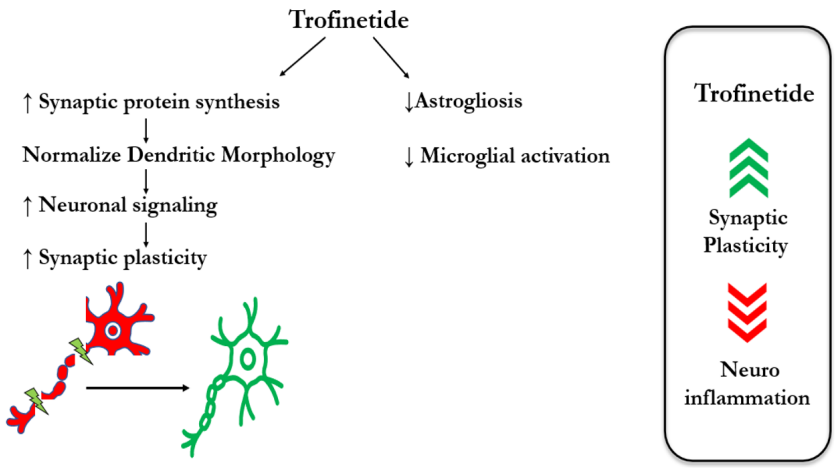

Figure 1. Proposed mechanism of action of Trofinetide.

unchanged by the renal route and, to some extent, by feces. Trofinetide is not recommended in patients with moderate or severe renal impairment (*16*).

4. Clinical trials

In an exploratory double-blind, placebo-controlled Phase II clinical trial, the safety, and efficacy of Trofinetide in adolescent and adult females with RTT were evaluated (*17*). The study enrolled 56 patients who were randomly assigned to receive either Trofinetide or placebo and divided into three cohorts, *i.e.*, 35mg/kg of Trofinetide twice daily for 14 days or placebo, 35 mg/kg Trofinetide twice daily for 28 days of placebo, and 70 mg/kg of Trofinetide twice daily for 28 days or placebo. The study's primary objective was to assess the efficacy, safety, and tolerability of Trofinetide in patients with RTT, and a p-value < 0.2 was considered indicative of efficacy. Out of three cohorts, the cohort with Trofinetide (70 mg/kg) showed significant improvement in three core parameters, *i.e.*, clinician-completed CGI-I, MBA change index, and caregiver-completed top-3 concerns. The results indicated that the 35 mg/kg and 70 mg/kg dose levels of Trofinetide were generally safe and well-tolerated, with no serious adverse events observed. The estimates are presented in Table 1. The study's conclusion suggests that Trofinetide is a safe and potentially effective treatment option for RTT patients (*17*).

Another double-blind, placebo-controlled, parallel-group trial evaluated the safety, tolerability, pharmacokinetics, and efficacy of Trofinetide in female children and adolescents with RTT (*18*). A total of 82 participants aged 5–15 years received a placebo twice daily for 14 days, followed by either placebo or one of three doses of Trofinetide (50, 100, or 200 mg/kg) twice daily for 42 days. The important efficacy endpoints were the Rett Syndrome Behavior Questionnaire (RSBQ), the Clinical Global Impression of Improvement (CGI-I), and the Rett Syndrome Domain Specific Concerns Questionnaire (RTT-DSC). Trofinetide was safe and well-tolerated at all three doses, with no deaths occurring during the study. Four serious adverse events (SAEs) occurred in three participants, with only one participant receiving Trofinetide, and all SAEs were considered unrelated to the study medication and resolved by the end of the study. The 200 mg/kg bid dose improved core neurobehavioral RTT symptoms, overall clinical status, and the most concerning aspects of RTT identified by clinicians. Details are presented in Table 1. Trofinetide also improved clinically significant symptom areas core to RTT, including the RTT-DSC domains of ambulation and seizures. These results prove that Trofinetide is safe, well-tolerated, and effective in improving RTT symptoms in children and adolescents (*18*).

In a pivotal phase III 12-week, randomized, double-blind, placebo-controlled clinical trial LAVENDER study, 187 girls and young women with RTT were

Table 1. Salient features of clinical trials of Trofinetide

Trial description (*Ref.*)	Age group, years	Comparators	Efficacy	Safety
Glaze DJ, *et al.* (*17*)	15.9–44.2	Trofinetide 70 mg/kg and 35 mg/kg twice daily or placebo	MBA change index: -2.01 *vs.* -0.62 (p = 0.146) CGI-I score: 3.24 *vs.* 3.64 (p = 0.164) Caregiver Top 3 Concerns total VAS score: -62.59 *vs.* -23.71 (p = 0.076) In this trial p <0.2 was considered significant.	• The most common encountered TEAE was diarrhoea (39% -35 mg/kg group *vs.* 15% - placebo group), irritability (22% -35 mg/kg group *vs.* 15% - placebo group), and somnolence (17% -70 mg/kg group *vs.* 5% - placebo group) • Most AEs were mild or moderate in intensity and most events were considered not related to study drug *SAE:* Three subjects experienced serious adverse events, 2 subjects in the 35 mg/kg cohort and 1 subject in the 70 mg/kg cohort. All SAEs were resolved by the end of the study.
Glaze DJ, *et al* (*18*)	5–15	Trofinetide 200 mg/kg twice daily *vs.* placebo.	RSBQ total score: -6.7 *vs.* -2.3; p = 0.042 CGI-I: 3 *vs.* 3.5; p = 0.029) RTT-DSC: -76 *vs.* 25.85; p = 0.025)	• The tolerability of Trofinetide was very good at all 3 dose levels • Diarrhoea: 200 mg (56%); 100 mg (13%), 50 mg (27%), placebo (4%) • Vomiting: 200 mg (22%); 100 mg (13%), 50 mg (7%), placebo (13%) • Upper respiratory tract infection: 200 mg (19%); 100 mg (0%), 50 mg (7%), placebo (13%) • Pyrexia: 200mg (11%); 100mg (19%), 50mg (0%), placebo (8%) *SAE:* Four SAEs occurred in 3 participants. All the SAEs were deemed not related to study medication and resolved by the end of the study.
Neul LJ, *et al* (*19*)	5–20	Trofinetide oral solution (1 g Trofinetide per 5 mL, calculated as per the weight) *vs.* placebo	RSBQ: -4.9 *vs.* -1.7; p = 0.018 CGI-I: 3.5 *vs.* 3.8; p = 0.003 CSBS-DP-IT Social total score: -0.1 *vs.* -1.1; p = 0.006	• Common treatment-emergent adverse events included diarrhea 80.6% for Trofinetide *vs.* 19.1% for placebo and vomiting 26.88% for Trofinetide *vs.* 9.57% for placebo • All the TEAE were of mild to moderate in severity *SAE:* 1 case of Bacteremia, bronchiolitis, covid-19 pneumonia, urinary tract infection, seizure was reported.

RSBQ: Rett Syndrome Behavior Questionnaire; CGI-I: Clinical Global Impression Scale-Improvement; RTT-DSC: RTT-Clinician Domain Specific Concerns; MBA: Motor Behavior Assessment scale; CSBS-DP-IT: Social Communication and Symbolic Behavior Scales Developmental Profile Infant-Toddler Checklist - Social Composite Score; TEAE: Treatment Emergent Adverse Event.

included. In this study, Trofinetide demonstrated statistically significant and clinically meaningful results over placebo and was effective and safe in treating RTT. Details are presented in Table 1. The findings from this trial have significant implications for the medical community. They could lead to the approval of Trofinetide as a treatment for RTT, addressing an unmet medical need for individuals with this debilitating disorder (*19*). The efficacy and safety endpoints of the various clinical trials of Trofinetide have been depicted in Table 1.

5. Place of Trofinetide in therapeutics and conclusion

RTT is a progressive neurodevelopmental disorder that affects females, and there is no specific therapy for RTT; managing associated conditions and addressing psychosocial function, including that of the family and caregivers, can improve the quality of life of RTT patients (*20*). A multidisciplinary approach is crucial to address nutritional, gastrointestinal, and motor problems, as well as nonepileptic behaviors and seizures.

Trofinetide is the first drug approved for this rare genetic disorder. It is approved for both adults and pediatric patients aged > 2 years. Trofinetide should be administered twice daily as per the weight band, *i.e*., 5,000 mg (9–12 kg), 6,000 mg (12–20 kg), 8,000 mg (20–35kg), 10,000 mg (35–50 kg),12,000 mg (≥ 50 kg). Trofinetide has been generally well-tolerated in clinical trials. Gastrointestinal symptoms, *i.e*., diarrhea and vomiting, are the most common adverse events associated with Trofinetide (*17-19*). Trofinetide should be discontinued in case of severe diarrhea. Trofinetide can also cause weight loss and should be stopped in case of significant weight loss. Other common adverse events were irritability, somnolence, and pyrexia. These symptoms usually occur early in treatment and can be managed with dose adjustments or symptomatic treatment. To the best of the available evidence, there are minimal drug-drug interactions that are likely to increase acceptability.

Various trials showed that Trofinetide demonstrated statistically significant clinical improvement in symptom areas core to RTT, and all the trials included patients with confirmed *MeCP2* gene mutation. Presently it is challenging to comment upon the efficacy of Trofinetide on various types of mutations giving rise to RTT. Furthermore, the trials have not presented the results per the different mutations. The exact place of Trofinetide managing RTT is awaited as the drug was recently approved. Future real-world studies will only tell us about the exact effectiveness and safety of Trofinetide. These findings suggest that Trofinetide may be an effective and safe treatment option for individuals with RTT. However, careful monitoring and individualized treatment plans are necessary to ensure the best possible outcomes.

Funding: None.

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

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Received July 21, 2023; Revised October 31, 2023; Accepted November 15, 2023.

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Released online in J-STAGE as advance publication November 18, 2023.

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DOI: 10.5582/irdr.2023.01094

Pathologic features and clinical treatment of sarcomatoid intrahepatic cholangiocarcinoma

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SUMMARY The current study examined sarcomatoid intrahepatic cholangiocarcinoma (S-iCCA). S-iCCA was a more aggressive subtype of intrahepatic cholangiocarcinoma (iCCA). Early detection and complete resection of tumors are very important. Reported here is a case of S-iCCA, and the diagnosis and treatment of S-iCCA are discussed. The patient underwent a tumor resection and was treated with chemotherapy and molecularly targeted drugs after surgery. The clinical pathologic features and treatment of S-iCCA are discussed based on the literature. An immunohistochemical examination revealed positivity for cytokeratin 7 (CK7), CK-pan, vimentin, and CK19 and negativity for hepatocyte paraffin 1 (HepPar-1) in sarcomatoid cells. This case suggests that the particular molecular characteristics of sarcomatoid cells have great clinical diagnostic value, and comprehensive treatment of S-iCCA based on surgery is described.

Keywords sarcomatoid intrahepatic cholangiocarcinoma, diagnosis, molecular pathology, surgery/drug therapy

Sarcomatoid carcinoma has been reported in various sites, including the upper digestive tract, lungs, pancreas, skin, breasts, thyroid, uterus, urinary tract, and gallbladder, and the most common organs are the breasts and lungs (*1,2*). Sarcomatoid intrahepatic cholangiocarcinoma is defined by the World Health Organization as iCCA with sarcomatoid changes (*3*). The mechanism of its pathogenesis is still unknown. Primary liver sarcomatoid carcinoma is rare, and most cases are hepatocellular sarcomatoid carcinoma (*4-6*). S-iCCA is an aggressive carcinoma with a high mortality and poor survival rate (*7,8*). Macroscopic vascular invasion, positive surgical margins, and an advanced TNM stage are associated with a high recurrence rate and a poor prognosis (*9*).

Reported here is a case involving a 68-year-old Chinese man who was admitted to this Hospital for intermittent right upper abdominal pain and a fever for 14 days. Half a month ago, the patient developed right upper quadrant pain of unknown origin, chills, a high fever, and a peak body temperature of 39.5°C, accompanied by nausea, vomiting, and diarrhea. Routine blood work and liver function were checked, and a hepatobiliary B-ultrasound and enhanced MRI were performed at a local hospital. The man was diagnosed with "massive hepatocellular carcinoma in the right lobe of the liver with intrahepatic bleeding, necrosis, and infection and multiple hepatic cysts". The man was treated with anti-inflammatories, antidiarrheals, and nutritional support, and his symptoms improved. The man then visited this Hospital for further diagnosis and treatment. The patient had an unremarkable medical and family history, a physical examination revealed tenderness in the right upper abdomen, and a 4 × 6 cm mass was palpable under the right costal margin; the mass was hard and fixed. After admission, routine laboratory tests, including serum glutamic oxaloacetic transaminase (AST), glutamic pyruvic transaminase (ALT), and the bilirubin index, were normal. Viral serology tests for hepatitis B surface antigen (HBsAg), hepatitis B E antigen (HBeAg), hepatitis C antibody, and hepatitis C virus (HCV) were negative. However, hepatitis B surface antibody, hepatitis B E antibody, and hepatitis B core antibody were positive. Hepatitis B virus DNA was < 100 IU/mL (normal range: < 100 IU/ mL). A serum tumor biomarker test revealed that the cancer antigen 125 (CA125) level was 69 U/mL (normal range: < 35 U/mL), while alpha-fetoprotein (AFP) and cancer antigen 19-9 (CA19-9) levels were normal.

A color Doppler ultrasound revealed an irregular and slightly hyperechoic region in the right lobe of the liver. The tumor was 14.1 × 10.5 cm in size, it had

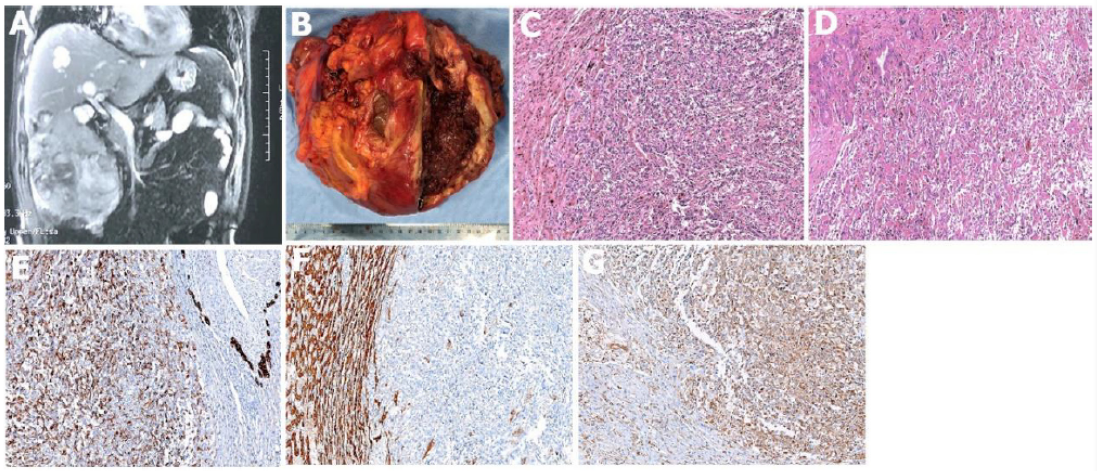

Figure 1. Clinical and pathologic findings of this study. (A) An enhanced MRI revealed an exophytic mass located in right liver segments 5 and 6, and mixed intensity lesions in the right lobe of the liver with uneven enhancement in the arterial phase. The tumor was about 15 × 13 × 11 cm in size. **(B)** Surgical specimen including the gallbladder, hepatic flexure, and tumor. It was about 23 × 17 × 16 cm, and grayish white, and hard. Intratumoral bleeding and necrosis were present. **(C)** A histopathological examination revealed sarcomatoid carcinoma (hematoxylin-eosin staining, ×10 original magnification). **(D)** A histopathological examination revealed the transition zone between sarcomatoid carcinoma and cholangiocarcinoma (hematoxylin-eosin staining, ×10 original magnification). **(E)** An immunohistochemical examination revealed positivity for CK19 in the cytoplasm of sarcomatoid cells (×10 original magnification). **(F)** An immunohistochemical examination revealed positivity for HepPar1 in the cytoplasm of paraneoplastic hepatocytes (×10 original magnification). (G) An immunohistochemical examination revealed positivity for vimentin in the cytoplasm of sarcomatoid cells (×10 original magnification).

distinct margins, and the internal echo was not uniform. A CDFI scan revealed obvious blood flow signals in the tumor. A contrast-enhanced MRI revealed signals of mixed intensity in the tumor in the right lobe of the liver, uneven enhancement in the arterial phase, and decreased enhancement in the delayed phase. The tumor was 14.1 × 9.6 cm in size. Several hyperintensities were noted in the liver, so primary liver cancer and multiple hepatic cysts were considered (Figure 1A).

The preoperative diagnosis was right lobe liver cancer and hepatic cysts. An exploratory laparoscopy was performed and revealed an exophytic mass located in right liver segments 5 and 6. The tumor was about 15 ×13 ×11 cm in size. The tumor involved the adjacent gallbladder and hepatic flexure of the colon with indistinct margins. A small volume of ascites was found in the abdominal cavity; it was light yellow and transparent. Exfoliative cytology of the ascites revealed no tumor cells during surgery. No enlarged lymph nodes were found in the hilar and duodenal ligaments during surgery. Resection of the right liver tumor, gallbladder and hepatic flexure of the colon was performed. The resected tumor specimen was off-white, bleeding, and necrotic, and the tumor was very hard. The gallbladder and hepatic flexure of the colon were closely attached to the tumor and not well demarcated from the tumor. The specimen, including the gallbladder, colon, and tumor, was about 23 ×17 × 16 cm in size (Figure 1B).

A histological examination of the tumor using hematoxylin and eosin staining revealed the presence of poorly-moderately differentiated S-iCCA. Tumor cells were found in the mucosa and serosa of the gallbladder and the serosa of the colon. No metastasis was found in the hilar lymph nodes. No tumor cells were found at the resection margin of the liver. A separate inspection revealed emboli in the blood vessels of cancer tissue, while the microvascular invasion (MVI) grade was M1. The tumor stage was determined to be T4N0M0 based on the 8th edition of the American Joint Committee on Cancer TNM staging system (*10*).

The tumor tissue consisted of different amounts of cholangiocarcinoma and sarcomatoid components, with bleeding and necrosis. The adenocarcinoma portion of the cholangiocarcinoma was poorly to moderately differentiated, and the sarcomatoid portion had a spindle cell and pseudohemangiomatous morphology. The tumor cells displayed infiltrative growth involving the surrounding liver tissue, moderate inflammation in the portal area of the liver tissue around the tumor, and obvious hyperplasia or dysplasia of small bile ducts (Figure 1C, D). An immunohistochemical examination of the tumor tissue revealed positivity for cytokeratin (CK) 7, CK-pan, CK19, and vimentin in the cytoplasm of sarcomatoid cells, positivity for hepatocyte paraffin 1 (HepPar-1) in the cytoplasm of paraneoplastic hepatocytes, and negativity for HepPar-1 in tumor cells (Figure 1E, F, G). The Ki-67 proliferation index was approximately 70%. Based on these histopathological and immunohistochemical findings, the patient was definitively diagnosed with S-iCCA.

Six cycles of chemotherapy were performed with the GS regimen (Gemcitabine plus S-1) in the third week after surgery, combined with the tyrosine kinase inhibitor anlotinib hydrochloride (12 mg, once a day for 2 weeks, respite for 1 week). Nonetheless, the patient died of intrahepatic recurrence and metastasis 8 months after surgery.

S-iCCA is defined by the World Health Organization

(2010) as "cholangiocarcinoma with a spindle cell component, such as spindle cell sarcoma, fibrosarcoma, or malignant fibrous histiocytoma; scattered cancer foci, including squamous cell carcinoma, can be seen within the tumor" (*11*). Sarcomatoid transformation was found in 3.9-9.4% of hepatocellular carcinomas and 4.5% of intrahepatic cholangiocarcinomas (*5,12*). Studies have shown that tumors have epithelial and heterologous mesenchymal components and have a subtle transition. The mesenchymal component has characteristics of epithelial tissue, such as similar gene and protein phenotypes, through metaplasia into mesenchymal cells and other characteristics (*13*).

The early symptoms of S-iCCA patients lack specificity, hampering diagnosis. S-iCCA is highly malignant because most of the tumors have spread to the liver, invaded adjacent organs, or metastasized to distant organs at the time of diagnosis. The surgical resection rate is low, the postoperative tumor recurrence and metastasis rates are high, and patient prognosis is poor (*14,15*). The diagnosis of S-iCCA requires pathological and immunohistochemical analysis. The tumor cells of S-iCCA mainly consist of spindle cells, giant cells and atypical cells, and malignant epithelial bile duct cells; sarcomatoid biphasic components and mixed phenotypic regions usually coexist. The sarcomatoid components of spindle cells are arranged in sheets or bundles and mixed with heterogeneous polymorphic giant cells. Epithelial tumor markers (CK, Keratin, and EMA) and mesenchymal tumor markers (Vimentin, SMA, and CD68) are simultaneously positive. Intermediate keratin (CAM5.2 and CK19) is positive (*16*), which is consistent with the immunohistochemical results in the current case. The presence of malignant epithelial bile duct cells and sarcomatoid biphasic components, positivity for epithelial tumor markers and mesenchymal tumor markers, and the presence of desmosome junctions between sarcomatoid cells are the characteristic pathological manifestations of S-iCCA and the gold standard for the diagnosis of S-iCCA.

Differential diagnosis can be made by carefully looking for epithelial components and the transition between epithelial components and sarcomatoid components. Histologically, S-iCCA needs to be differentiated from the following tumors: *(i)* S-iCCA is mainly associated with hepatocellular differentiation of sarcomatoid carcinoma and mixed hepatocellular-biliary sarcomatoid carcinoma. With the former, the level of positivity for HepPar1 differs and it occasionally has hepatoblastoma features, which increases the difficulty of diagnosis (*6*); with the latter, HepPar 1 and CK19 are simultaneously positive (*17*). *(ii)* S-iCCA needs to be differentiated from primary sarcoma (including angiosarcoma, undifferentiated embryonal sarcoma, and malignant teratoma). Angiosarcoma is positive for CD31 and CD34 and negative for CK. In addition to the characteristic histological morphology, malignant teratoma can express various markers of mesenchymal differentiation at different levels. Residual hepatocytes and bile duct components can be seen in the tumor, but primitive cells expressing high and low molecular weight CK, which may be differentiated from primitive stem cells are also observed (*18*). *(iii)* S-iCCA needs to be distinguished from epithelioid or exotic angiomyolipomas, angiomyolipomas with significant atypia, which are positive for HBM45 and SMA. *(iv)* S-iCCA needs to be differentiated from metastatic carcinoma and metastatic mesenchymal tumors, which usually have a clear medical history of a primary tumor. The corresponding epithelial markers may be expressed in metastatic carcinoma, and metastatic mesenchymal tumor are mainly positive for characteristic markers of gastrointestinal stromal tumors and melanoma.

Although there are few treatments for S-iCCA and their effectiveness is limited, surgical resection is still the preferred treatment. For inoperable tumors, chemotherapy, molecularly targeted drugs, and immunotherapy (such as PD-1/PD-L1) are still in the clinical exploratory stage.

In conclusion, S-iCCA is too difficult to diagnose early through a clinical examination and imaging studies, and it can easily be misdiagnosed as a liver abscess, liver metastasis, or the like. Assuming that it could be diagnosed early and treated surgically, the patient a better clinical prognosis. The determination of S-iCCA is based on some important findings: *(i)* a history of intrahepatic bile duct stones and recurrent bile duct infections, *(ii)* imaging studies and blood tumor markers heavily suggest sarcoma and require a pathological biopsy, *(iii)* positivity for epithelial tumor markers (CK, Keratin, and EMA) and mesenchymal tumor markers (Vimentin, SMA, and CD68) in the cytoplasm of sarcomatoid cells, *(iv)* positivity for intermediate keratin (CAM5.2) in the cytoplasm of sarcomatoid cells, and positivity for HepPar1 in the cytoplasm of paraneoplastic hepatocytes.

Acknowledgements

The authors would like to thank Dr. Han Wang (Department of Pathology, The Third Hospital Affiliated with Naval Military Medical University, Shanghai, China) for helpful advice and discussion.

Funding: This study was supported in part by a grant-in-aid for a Minhang Project of the Shanghai Science and Technology Commission (2021MHZ065, 2022MHZ089), the Minhang District of Shanghai Commission to Create Medical Specialties (2020MWFC03), and the Shanghai Minhang Hospital Project to Create Departments (YJXK-2021-09-003).

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

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Received October 2, 2023; Revised November 5, 2023; Accepted November 8, 2023.

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Released online in J-STAGE as advance publication November 10, 2023.

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

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

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

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

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

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

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

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

3. Editorial Policies

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

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

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

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

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

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

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The manuscript must be accompanied by a cover letter prepared by the corresponding author on behalf of all authors. The letter should indicate the basic findings of the work and their significance. The letter should also include a statement affirming that all authors concur with the submission and that the material submitted for publication has not been published previously or is not under consideration for publication elsewhere. The cover letter should be submitted in PDF format. For an example of Cover Letter, please visit: (Download Centre: *https://www.irdrjournal.com/downcentre*)

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6. Manuscript Preparation

Manuscripts are suggested to be prepared in accordance with

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

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

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

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Introduction: The introduction should provide sufficient background information to make the article intelligible to readers in other disciplines and sufficient context clarifying the significance of the experimental findings.

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Acknowledgments: All funding sources (including grant identification) should be credited in the Acknowledgments section. Authors should also describe the role of the study sponsor(s), if any, in study design; in the collection, analysis, and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication. If the funding source had no such involvement, the authors should so state.

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Examples are given below:

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

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

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

Example 3 (Sample book reference):
Shalev AY. Post-traumatic stress disorder: Diagnosis, history and life course. In: Post-traumatic Stress Disorder, Diagnosis, Management and Treatment (Nutt DJ, Davidson JR, Zohar J, eds.). Martin Dunitz, London, UK, 2000; pp. 1-15.

Example 4 (Sample web page reference):

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

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

Figure Legend: The figure legend should be typed on a separate page of the main manuscript and should include a short title and explanation. The legend should be concise but comprehensive and should be

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(As of September 2023)

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