Vol. 6 • No. 2 • October 2024
http://isgm.kr
eISSN 2671-6771

JIG

Journal of Interdisciplinary Genomics

Interdisciplinary Society of Genetic & Genomic Medicine

JIG Journal of Interdisciplinary Genomics

http://isgm.kr

eISSN 2671-6771

Vol. 6, No.2, October 2024
Published in 31 October 2024

AIMS AND SCOPE

Journal of Interdisciplinary Genomics (J Int Gen; JIG, http://accesson.kr/jig) is an official publication of the Interdisciplinary Society of Genetic & Genomic Medicine (ISGM; http://isgm.kr/). The journal is devoted to the dissemination the understanding of knowledge on the field of cytogenetics, molecular genetics, and clinical genetics through sharing of the latest scientific information.

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Publisher: **Chong Kun Cheon**
Published by **Interdisciplinary Society of Genetic & Genomic Medicine**
Editor-in-Chief: **Kyung Ran Jun**

Editorial Office: Interdisciplinary Society of Genetic & Genomic Medicine
Room 3206, Building A, 160 Chungjang-daero, Dong-gu, Busan 48751, Korea
Tel: +82-55-366-7716, Fax: +82-55-360-2181, Homepage: http://isgm.kr
E-mail: jun@paik.ac.kr (Editor-in-Chief)

Printed by Academya Publishing Co.
Room 2003, Daerung Techno Town 15-Cha, 401 Simin-daero, Dongan-gu, Anyang 14057, Korea
Tel: +82-31-389-8811, Fax: +82-31-389-8817, E-mail: journal@academya.co.kr, Homepage: https://academya.co.kr/

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eISSN 2671-6771

Vol. 6, No. 2, October 2024

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https://doi.org/10.22742/JIG.2024.6.2.15
Journal of Interdisciplinary Genomics 2024;6(2):15-20
eISSN 2671-6771

The Growing Potential of Long-Read Sequencing in Identifying Previously Elusive Causative Variants in Patients with Undiagnosed Rare Diseases

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Rare diseases, largely driven by genetic factors, present significant diagnostic challenges due to their complex genomic variations. Traditional short-read sequencing methods, such as whole-exome sequencing and whole-genome sequencing, are widely used to detect genomic alterations in a time- and cost-effective manner. However, some rare conditions are often left undiagnosed due to the technical limitations of current sequencing platforms. To overcome these limitations, long-read sequencing (LRS) technology has been applied to various fields of clinical research including rare diseases. With LRS, researchers are able to accurately characterize complex variants such as structural variations, tandem repeats, transposable elements, and transcript isoforms. This review article explores the current applications of LRS in rare disease research, highlighting its potential in identifying previously elusive causative variants in undiagnosed rare diseases.

Key words: Long-read sequencing, Rare diseases, Mendelian disorder, Structural variations, Tandem repeats, Transposable elements, Transcript isoforms

REVIEW ARTICLE

Received: August 29, 2024
Revised: September 5, 2024
Accepted: September 12, 2024

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INTRODUCTION

Rare diseases, typically defined as conditions affecting fewer than 1 in 2,000 individuals, represent a significant global health challenge. It is estimated that over 400 million people worldwide suffer from such conditions, with approximately 80% of these diseases believed to be genetic in origin [1,2]. The Online Mendelian Inheritance in Man (OMIM) database currently documents over 6,300 distinct disease-gene associations, highlighting the genetic diversity and complexity inherent in rare diseases.

In South Korea, rare diseases are defined more narrowly, where conditions affecting fewer than 20,000 individuals are considered rare [3]. Among these, diseases with fewer than 200 patients are classified as extremely rare. The limited number of patients, along with the requirement of specialized knowledge for diagnosis, often complicates the identification and management of these diseases. As of November 2023, approximately 1,250 diseases are officially recognized as rare in South Korea, where the national healthcare system provides coverage for the associated medical expenses. This underscores the significant burden that rare diseases place on patients and healthcare systems alike, given the persistent health risks and complications associated with these conditions.

Currently, the most widely used next-generation sequencing (NGS) technology for diagnosis of rare diseases is whole-exome sequencing (WES). This method targets the exonic region, which contains the protein-coding sequences of the ge-

nome. Although the exome only comprises 1%–2% of the genome, it contains 85% of genetic variants that have a high impact on the pathogenicity of diseases [4]. In addition, WES allows for a high sequencing depth in an accurate and cost-effective manner compared to other sequencing platforms, enabling the reliable detection of genetic variants such as single nucleotide variants (SNVs) and short insertions and deletions (INDELs). Moreover, data generated by WES is relatively small and manageable, greatly reducing computational burden and time required for analysis. These factors make WES meritable for researchers with large patient cohorts and even make trio analysis feasible, which is very important in identifying causative variants.

Despite the advantages described above, WES is limited to the exonic region; therefore, non-coding variants and structural variations (SVs) in the intronic regions cannot be detected. Hence, whole-genome sequencing (WGS) is used when genome-wide analysis is necessary. SNVs and INDELs in the non-coding regions in addition to SVs can be detected with WGS, which can make this sequencing platform more appropriate than WES depending on the aim of the study. However, short-read WGS produces sequence reads with an average length of 100 bp. As such, analysis of complex SVs or repeat regions with lengths over 100 bp is still challenging.

Diagnosis rates using WES or WGS data range from 25%–50%, yielding slightly better rates with WGS [5,6]. In other words, roughly 50% of patients remain undiagnosed. Among many factors behind the difficulties hindering diagnosis, the technical limitations of short-read sequencing (SRS) contribute considerably by limiting the effectiveness in detecting and analyzing complex genetic variations. To address these issues, developments in long-read sequencing (LRS) have emerged recently and attempts to identify previously undetected causative variants have increased using this technology.

As of date, the most prevalent LRS platforms have been developed by PacBio and Oxford Nanopore Technologies (ONT), where each has its distinctions. PacBio's LRS technology is known for its overall high data quality, as molecules can be sequenced multiple times to generate low-error data [7]. However, LRS data generation with PacBio has a higher cost and requires larger amounts of higher-quality DNA [8]. On the other hand, ONT's LRS technology provides a higher throughput at a lower cost, which is an important factor for improving the efficiency and scalability of research projects. Furthermore, longer mappable reads are achievable with ONT, but generally shorter reads are generated compared to PacBio [9]. As such, both technologies have their differences and researchers should choose LRS platforms that are the most appropriate depending on the objectives of their research.

The introduction of LRS technology has enabled the characterization of complex SVs, repeat regions, and phases that were previously difficult to identify, as well as the characterization of gene isoforms [10]. On the other hand, LRS also comes with certain limitations, including high cost and low accuracy per read. Furthermore, current bioinformatic analysis methods for LRS are limited and are still under development. Nevertheless, LRS definitely has its strengths, and various efforts are continuously being made to minimize error and improve analysis techniques. With advancements in this technology, LRS has significant potential to help characterize the complex genomic changes occurring in rare diseases and accurately diagnose affected patients.

This review article aims to introduce LRS technology and outline the applications of LRS in identifying causative variants of mendelian disorders or rare diseases.

COMPLEX STRUCTURAL VARIATIONS

Structural variations (SVs), which are large genomic alterations with sizes ranging from 50 bp to over 1 kb, are commonly associated with genetic diseases [11]. These alterations include inversions, translocations, insertions, deletions and can have a significant impact on a gene's function. Due to their sizes, accurately depicting large SVs with SRS serves to be quite difficult. Furthermore, current NGS techniques have limited capabilities in detecting SVs in regions with complex or repeated sequences. To address this problem, researchers have adopted LRS to characterize these alterations effectively (Fig. 1A). With LRS, it is possible to survey longer portions of the genome and in turn, observe SVs with higher accuracy [12]. Merker et al. [13] performed low-coverage genome LRS to identify SVs that could not be detected using SRS in a patient with multiple neoplasia and cardiac myxomata. As a result, they identified over 6,000 insertions and deletions larger than 50 bp, including a pathogenic 2,184 bp deletion overlapping with an exon of the *PRKAR1A* gene, which is associated with autosomal dominant Carney complex. Similar studies have been performed using PacBio LRS as well as Nanopore LRS techniques. In a study by Damián et al. [14], LRS was performed on two patients with congenital aniridia for whom causative variants were not identified through SRS analysis. This approach revealed pathogenic SVs in the *PAX6* gene responsible for con-

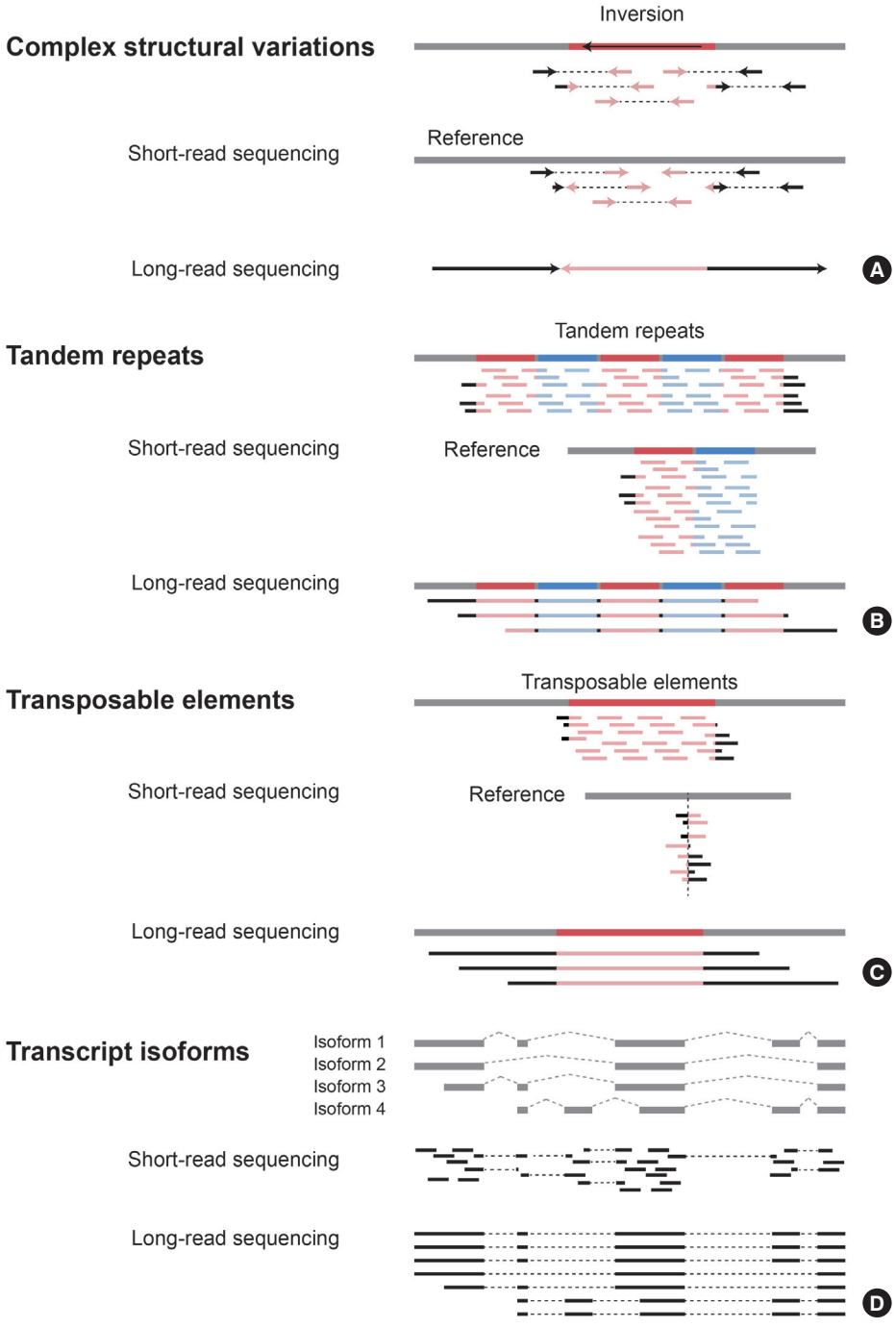

Fig. 1. Identifying causative variants using short-read sequencing vs. long-read sequencing. (A) Detection of complex structural variations. Diagram showing an example of sequencing complex structural variations. The black and light red arrows indicate reads mapped to the complex structural variation. (B) Detection of tandem repeats. The colored regions indicate repeats occurring in the genome and different colors were used to differentiate each repeat unit. The light red and light blue lines show reads mapped to the repeat region. (C) Detection of transposable elements. The red regions indicate transposable elements. The light red lines show reads mapped to the transposable element region. (D) Detection of transcript isoforms. The black regions are reads mapped to the transcripts.

genital aniridia, including a 4.9 Mb inversion in intron 7 and a t(6;11) balanced translocation. In another study, long-read WGS was performed on a patient suspected of having autosomal recessive glycogen storage disease type Ia (GSD-Ia) in whom the causative variant had not been properly identified by WES. While WES had detected only a single heterozygous

pathogenic variant in the *G6PC* gene, LRS revealed the presence of a 7.1 kb deletion covering two exons on the other allele (Miao et al.) [15]. Furthermore, there are studies where LRS has been performed on a larger cohort as opposed to a few samples. Miller et al. conducted targeted LRS on 30 patients with previously identified causative variants and 10 patients without an accurate diagnosis. They successfully confirmed the previously identified pathogenic SVs and further detected pathogenic/likely pathogenic variants in 6 out of the 10 undiagnosed patients [16]. These studies demonstrate the potential of LRS in characterizing complex SVs, especially those that could not be identified with conventional SRS methods.

TANDEM REPEATS

Tandem repeats, which are short copies of DNA sequences that are repeated multiple times in the genome, are highly polymorphic and are an important source of genetic variation [17]. The size of these repeated sequences range from a few base pairs to hundreds of base pairs. In the case of SRS, the read length is approximately 100 bp, rendering large tandem repeats difficult to be mapped accurately to the genome. On the other hand, LRS allows the detection of repeated sequences in large regions, which makes it suitable for tandem repeat analysis (Fig. 1B). In a study by Mizuguchi et al. [18], LRS was used to precisely investigate the causative variants in a benign adult familial myoclonus epilepsy (BAFME) family. As a result, they found a 4,661 bp heterozygous repeat insertion in the *SAMD12* intron region. Also, LRS from ONT was used to identify repeat expansions. With this approach, Sone et al. [19] confirmed that the 5' GCC repeat expansion in *NOTCH2NLC* was found only in neuronal intranuclear inclusion disease family members. These findings highlight the advantages of using LRS technology to provide deeper insight into complex diseases through accurate detection of tandem repeats and repeat expansions.

TRANSPOSABLE ELEMENTS

Transposable elements (TEs) have also been reported to play a role in rare diseases [20]. TEs are also known as "jumping genes," and as the name suggests, they are mobile DNA sequences that can move to different genomic locations. They can be found throughout all living organisms and are another source of evolution and genome reorganization. TE integration in the protein-coding region can induce gene dysfunction, while integration in the intronic region can induce alternative splicing events and in turn, abnormal gene expression [21]. A constraint of short-read WGS is that the generated reads are too short to fully cover each TE copy. Thus, only single nucleotide polymorphisms within a TE, or partial reads containing TE and genome junctions, can be mapped. Conversely, LRS overcomes this obstacle by generating reads of sufficient length to identify TE insertions (Fig. 1C) [22]. Zhou et al. [23] identified 90 L1Hs insertions in human cell lines that were not detected by previous SRS studies by developing computational software (PALMER) to detect LINE-1 insertions from PacBio reads. Moreover, LRS can be used in conjunction with other genomic analysis methods for TE analysis. Aneichyk et al. [24] also used multiple approaches, including PacBio SMRT in a X-Linked Dystonia-Parkinsonism (XDP) cohort to analyze the cause of the disease. They identified an SVA (SINE-VNTR-Alu) insertion in the *TAF1* gene that was exclusive to the XDP probands. In a different study, Fernández-Suárez et al. [25] employed LRS to detect an Alu retrotransposon insertion in the *EYS* gene from patients with retinitis pigmentosa, which was not discovered with their previous targeted SRS results. With LRS, researchers are able to discern genomic changes that span the genome in broad segments, which can contribute to fully characterizing presently ambiguous regions of the genome.

TRANSCRIPT ISOFORMS

Transcript isoforms, which are different versions of mRNA produced from the same gene, represent another factor contributing to the genetic alterations that can lead to rare diseases [26]. These mRNA sequences are produced as a result of alternative splicing events, which can cause the synthesis of abnormal transcripts or change the expression levels of otherwise normally produced genes. With SRS, identifying and analyzing the transcript isoforms coded by genes usually required the prediction of these various isoforms using average read depth. In contrast, LRS can sequence whole RNA molecules, and can detect novel isoforms, along with their expression levels (Fig. 1D). In a study by Stergachis et al. [27], they performed full-length long-read isoform sequencing to establish the consequence of a *MFN2* intron branch point variant. They confirmed that the variant produces five altered splicing transcripts that disrupt the open reading frames responsible for Charcot-Marie-Tooth disease, axonal, type 2A (CMT2A). This illustrates that LRS is not limited to DNA and can be applied to research that requires detection of transcriptomic isoforms.

CONCLUSION

In this review article, we introduced the ongoing research on the applications of LRS technology in effectively identifying genomic variations that previously could not be detected with conventional methods. While acknowledging the current limitations of LRS, it is important to recognize the advantages that are accompanied. With further improvements in the LRS platform and bioinformatic methodology, we believe that this technology has the potential to expand our knowledge on the complex mechanisms underlying rare diseases, ultimately aiming at improved patient diagnosis and appropriate treatment.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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JIG Journal of Interdisciplinary Genomics

https://doi.org/10.22742/JIG.2024.6.2.21
Journal of Interdisciplinary Genomics 2024;6(2):21-24
eISSN 2671-6771

Searching for the Missing Kallmann Syndrome Gene at 9q31.3

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The disease gene for delayed puberty is hypothesized to reside within a 3.7 Mb genomic region on chromosome 9, spanning 9q31.2 to 9q31.3, which contains 20 genes. This region aligns with 9q31.3, where the Kallmann syndrome gene is suspected to be located in a patient with a *de novo* balanced translocation, t(7;9)(p14.1;q31.3). After analyzing the expression patterns and reported genetic variants of the 20 candidate genes, we propose *ACTL7A* and *ACTL7B* as strong candidate genes for Kallmann syndrome. Mutation screening of these genes in Kallmann syndrome patients will be essential to confirm their pathological roles in delayed puberty.

Key words: Kallmann syndrome, 9q31.3, Balanced translocation, *ACTL7A, ACTL7B*, Delayed puberty, t(7;9)(p14.1;q31.3)

REVIEW ARTICLE

Received: August 30, 2024
Revised: October 1, 2024
Accepted: October 7, 2024

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INTRODUCTION

A study identified a 4.78 Mb familial heterozygous microdeletion at 9q31.2-q31.3, segregating across three generations. This deletion, encompassing 26 genes, is associated with delayed puberty in both sexes between ages 13 and 16. Through genomic analysis, the critical region for delayed puberty was refined to a 3.7 Mb spanning 9q31.3 to 9q32 [1]. Taking advantage of an overlooked *de novo* balanced translocation in a Kallmann syndrome patient [2]—where delayed puberty is a hallmark feature—we further narrowed the candidate region to 9q31.3 and identified two potential Kallmann syndrome genes based on gene expression patterns.

SEARCHING FOR THE MISSING KALLMANN SYNDROME GENE

Positional cloning is a powerful method for identifying disease genes, particularly when it capitalizes on chromosomal rearrangements such as deletions and balanced translocations. These rearrangements serve as invaluable tools to pinpoint the exact location of disease-causing genes. By providing clues about the chromosomal position where the gene resides, it enables researchers to focus on a specific genomic area and use it as a starting point to clone and identify the gene.

Deletions, which involve the loss of a specific chromosomal segment, are particularly informative. When a deletion is associated with a particular disease or phenotype, it strongly suggests that the missing genomic region contains the disease-causing gene(s).

Balanced translocations, on the other hand, involve the exchange of segments between two different chromosomes without any loss or gain of genetic material.

Despite this genomic balance, about six percent of carriers of balanced translocations exhibit abnormal phenotypes due to gene disruption at the breakpoints or dysregulation caused by position effects [3]. This dysregulation occurs when the separation of a gene from its cis-regulatory elements results in reduced gene expression [4]. By mapping these breakpoints, researchers can precisely identify candidate genes implicated in the disorder.

Contiguous deletion syndrome represents a genetic condition caused by a deletion of a segment of DNA that spans multiple genes located next to each other on a specific chromosome. The loss of these genes can lead to a variety of symptoms, depending on which genes are affected. Because multiple genes are involved, the resulting clinical features can be complex and variable, often including developmental delays, intellectual disabilities, congenital anomalies, and other distinctive phenotypes.

Comparative genomic mapping analyzes the chromosomal regions where microdeletions occur in different individuals who share a specific phenotype. By comparing these microdeletions, it is possible to identify overlapping genomic regions that are consistently missing across multiple cases. This overlapping region is considered critical because it likely contains the gene or genes responsible for the shared phenotype [5].

The process typically starts with identifying individuals who have similar clinical features or diseases and then performing detailed genetic analyses to map the precise locations of their chromosomal deletions. By narrowing down to the smallest common region deleted across these individuals, candidate genes within this region that might be causing the phenotype can be identified. This method has been successfully utilized to identify disease genes associated with various genetic syndromes, particularly by complementing next-generation sequencing, where interpreting the pathogenicity of variants of uncertain significance (VUS) remains challenging.

A novel 4.78 Mb familial heterozygous microdeletion segregating with the phenotype in three generation and encompassing distal end of 9q31.2 and entire 9q31.3 has been published in 2019. It contains 26 genes and is characterized by fatigue, muscle cramps, short stature, delayed puberty, sensorineural hearing loss (SNHL), and mild developmental delay. Male patients showed small testes, low testosterone levels and patients of both sexes showed delayed puberty from age 13 to 16 years old. Among the 25 genes within this genomic region, the authors proposed two candidate genes that may be associated with delayed puberty. They suggested that *UGCG*, located at 9q31.3, could be significant due to its role in the maturation of sperm-specific glycosphingolipids. Deletion of *UGCG* in

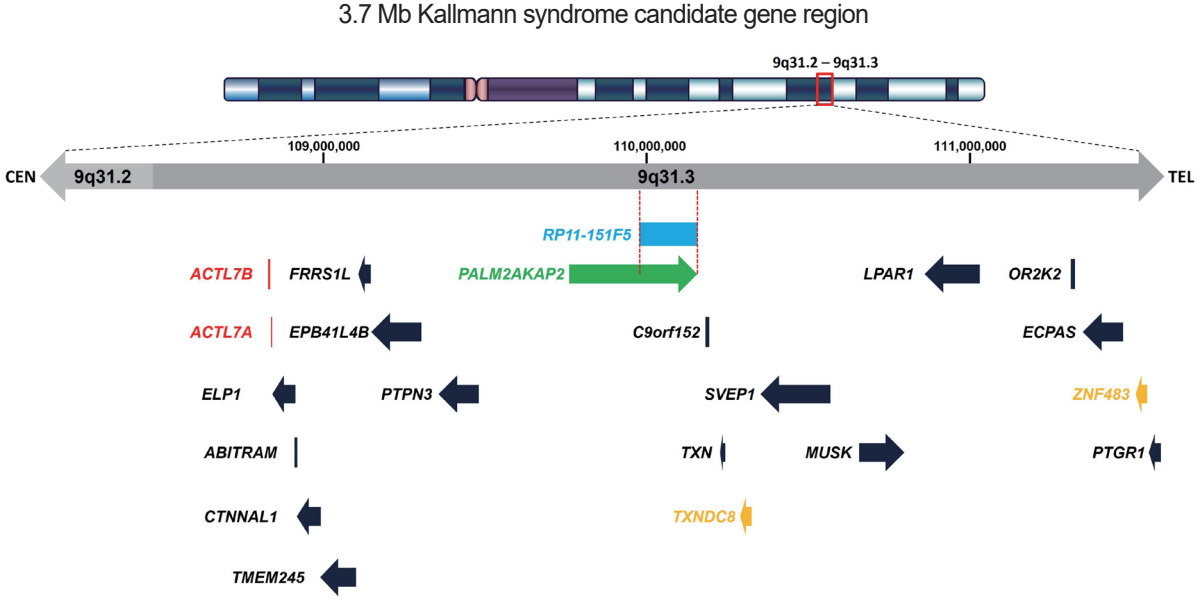

Fig. 1. The 3.7 Mb candidate gene region for Kallmann syndrome. This genomic region, refined by two heterozygous deletions, spans 20 genes located from 9q31.2 to 9q31.3 (GRCh38/hg38). Arrows indicate the transcriptional direction of each gene. The blue box represents the BAC clone RP11-151F5, identified by FISH to span the genomic breakpoint. This clone overlaps with a single gene *PALM2AKAP2*, shown in green, indicating that this gene is directly disrupted by the genomic breakpoint at 9q31.3 in a Kallmann syndrome patient with t(7;9)(p14.1;q31.3)dn. Two prime candidate genes for Kallmann syndrome are highlighted in red, while two additional genes discussed in this review and in the literature are shown in yellow.

mouse germ cells led to an age-dependent reduction in testicular mass, tubular atrophy, and arrested spermatogenesis. Additionally, they highlighted *ZNF483* at 9q31.3 as another potential candidate (Fig. 1), noting its association with the age at menarche in women [1].

Through comparative genomic mapping with another microdeletion case, a critical overlapping region of 3.7 Mb, spanning 9q31.3 to 9q32 (chr9: 107,909,771-111,605,520, hg38/chr9: 109,711,873-113,407,621, hg18), has been identified (Fig. 1). This region, which contains 20 genes, is expected to be associated with short stature, SNHL, pubertal delay, and developmental delay [1].

However, a *de novo* balanced translocation t(7;9)(p14.1;q31.3) in a patient with Kallmann syndrome, published in 2007, could significantly narrow down the candidate gene region at 9q31.3 for delayed puberty [2]. Kallmann syndrome is a genetic condition characterized by delayed or absent puberty and an impaired sense of smell (anosmia). It results from a failure in the development or migration of neurons responsible for producing gonadotropin-releasing hormone (GnRH), leading to insufficient production of gonadotropins and sex hormones with resultant delayed puberty [6]. In the cytogenetic characterization of a patient with KS and bone anomalies, demonstrating a balanced *de novo* translocation between chromosome 7 and chromosome 9, the authors hypothesized that the disease gene is located either 7p14.1 or 9q31.3 genomic breakpoints. Based on the delayed puberty found in two heterozygous deletions and its refined region 3.7 Mb, spanning 9q31.3 to 9q32, which encompasses 9q31.3, it is highly likely the disease gene for Kallmann syndrome should be at or in the vicinity of 9q31.3 breakpoint [1,7].

Using Bacterial Artificial Chromosomes (BACs) in a Fluorescent In Situ Hybridization (FISH) experiment, they found a176 kb BAC clone, RP11-151F5, located at 9q31.3. This clone resides within the extended and renamed gene *PALM2AKAP2* (previously annotated as separate genes, *PALM2* and *AKAP2*) [2]. The BAC spans exons 7 to 10 of *PALM2AKAP2* (NM_007203), indicating that the genomic breakpoint disrupts this gene (Fig. 1). Mutation screening in 98 Kallmann syndrome patients revealed no mutations, only two rare polymorphisms. This gene is expressed in olfactory epithelium and olfactory bulb [2], which are critical for the sense of smell and the migration of GnRH neurons during embryonic development. The olfactory epithelium contains sensory neurons that detect odors, and these neurons send signals to the olfactory bulb. During development, GnRH neurons originate in the olfactory epithelium and migrate to the hypothalamus via the olfactory bulb. Disruption in this pathway can lead to anosmia (loss of smell) and hypogonadotropic hypogonadism, the key features of Kallmann syndrome.

However, neither *PALM2* nor *AKAP2* is expressed in the testis, which is not necessarily incompatible with Kallmann syndrome, the genes of which are mostly expressed in the hypothalamus and/or pituitary, and the expression pattern of *PALM2AKAP2* is not available in the GTEx database (https://gtexportal.org/home/). Among the genes in the vicinity of *PALM2AKAP2* at 9q31.3, three genes-*TXNDC8*, *ACTL7A*, and *ACTL7B*-are notably predominantly expressed in the testis. *TXNDC8* is located 131 kb distal to *PALM2AKAP2*, while *ACTL7A* (Actin-like 7A) and *ACTL7B* (Actin-like 7B) are 777 kb and 785 kb proximal, respectively (Fig. 1). In GTEx, the median Transcripts Per Million (TPM) for *TXNDC8* is 12.57, *ACTL7A* is 543.2, and *ACTL7B* is 301.5. In a Northern blot analysis, *ACTL7A* is expressed across various adult tissues, with the highest expression observed in testis. In contrast, the *ACTL7B* transcript was detected exclusively in the testis, with a lesser extent in the prostate [8].

The protein encoded by this gene is a member of the actin-related proteins (ARPs) family, which shares significant amino acid sequence similarity with conventional actins [9]. Both actins and ARPs feature an actin fold, an ATP-binding cleft, which is highly conserved in *ACTL7A* and *ACTL7B*, suggesting potential ATPase activity. ARPs are involved in various cellular processes, including vesicular transport, spindle orientation, nuclear migration and chromatin remodeling [8]. *ACTL7A* and *ACTL7B* are intronless genes located approximately 6 kb apart at 9q31.3, in a head-to-head orientation with opposite transcription directions.

A homozygous missense mutation (p.Ala245Thr in NP_006678.1) in *ACTL7A* has been linked to infertility and early embryonic arrest in two consanguineous brothers [10], while compound heterozygous mutations (Arg155Ter and Gly362Arg) have been found in a non-consanguineous male with infertility [11]. Additional cases include a homozygous (Arg373Cys) and compound heterozygous mutations (Arg373His and Gly402Ser) identified in both consanguineous and non-consanguineous males with infertility [12]. Furthermore, a nonsense homozygous (Ser49Ter) and a missense homozygous mutations (Asp75Ala) were identified in two independent consanguineous males [13,14]. In a consanguineous Pakistani family with eight infertile men, a frameshift homozygous mutation (Glu50Alafs*6) was identified [15]. To date, no

mutations in *ACTL7B* have been reported in humans with reproductive phenotype.

Collectively, therefore, dysregulation of *ACTL7A* or *ACTL7B* due to position effect in a balanced translocation patient [4] or its haploinsufficiency in two heterozygous deletion patients may contribute to Kallmann syndrome. If either of these two genes is dysregulated due to a position effect, qRT-PCR analysis using blood RNA from a Kallmann syndrome patient with a balanced translocation would show a reduced transcript level compared to a control family member without chromosomal anomalies, assuming that these genes are expressed in blood.

Given that bi-allelic mutations in *ACTL7A* cause male infertility, it is unlikely that a heterozygous deletion or dysregulation of this gene in balanced translocation would lead to Kallmann syndrome. If this were the case, the parents of infertile males with bi-allelic mutations, who should be obligate carriers of the heterozygous mutations, would themselves exhibit delayed puberty or Kallmann syndrome, which was not observed. Moreover, due to their reproductive phenotype, they would likely face challenges in having children. This further suggests that a heterozygous deletion or dysregulation of the *ACTL7A* gene is unlikely to cause Kallmann syndrome. Screening for mutations in these two genes in Kallmann syndrome patients would help confirm their pathogenic roles in this reproductive phenotype.

CONCLUSION

By comparing the genomic positions of genes within the refined 3.7 Mb heterozygous region at 9q31.3 to 9q32 with those at the 9q31.3 breakpoint of a balanced translocation t(7;9)(p14.1;q31.3), we identified two candidate genes for Kallmann syndrome, *ACTL7A* and *ACTL7B*, at 9q31.3 based on their expression patterns.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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JIG Journal of Interdisciplinary Genomics

https://doi.org/10.22742/JIG.2024.6.2.25
Journal of Interdisciplinary Genomics 2024;6(2):25-32
eISSN 2671-6771

Exploring the Genetic Mechanisms Underlying Diamond-Blackfan Anemia

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Diamond-Blackfan Anemia (DBA) is a rare congenital bone marrow failure syndrome primarily characterized by erythroblastopenia and macrocytic anemia. This disorder results from mutations in ribosomal protein (RP) genes, which lead to defective ribosomal RNA maturation, nucleolar stress, and impaired erythropoiesis. Mutations in RP genes have been identified, with *RPS19* being the most commonly affected gene, accounting for approximately 25% of all cases. Other frequently mutated genes include *RPL5, RPL11*, and *RPS26*. These mutations are mostly heterozygous and cause defective ribosome assembly and biogenesis, which activates the p53 pathway, resulting in cell cycle arrest and apoptosis. In addition, non-RP gene mutations, such as those in *GATA1, TSR2,* or *HEATR3*, have been linked to DBA-like phenotypes, further complicating the genetic landscape. Congenital malformations, particularly craniofacial anomalies, thumb abnormalities, and cardiac defects, are common in patients with specific RP gene mutations, such as *RPL5* and *RPL11*. Advances in next-generation sequencing have improved the identification of novel mutations; however, approximately 20–25% of DBA cases remain genetically unexplained. In this review, we explore the genetic landscape of DBA and provide insights into the underlying mutations and their contributions to disease pathophysiology.

Key words: Diamond-Blackfan Anemia, Erythropoiesis, Ribosomal protein genes, p53 pathway

REVIEW ARTICLE

Received: September 12, 2024
Revised: October 5, 2024
Accepted: October 21, 2024

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INTRODUCTION

Diamond-Blackfan Anemia (DBA) is a congenital bone marrow (BM) failure syndrome characterized by increased susceptibility of erythroid progenitors and precursors to apoptosis, resulting in impaired erythropoiesis and subsequent erythropoietic failure [1]. The incidence of DBA is estimated to be approximately 7 cases per million live births, with most diagnoses occurring within the first year of life [1]. Clinically, DBA presents with congenital malformations in approximately 50% of patients, including craniofacial, skeletal, genitourinary, cardiac, and ophthalmologic anomalies [2]. Laboratory findings typically reveal normochromic macrocytic anemia, reticulocytopenia, and reduced levels of erythroid precursors in the BM, whereas other hematopoietic lineages are usually unaffected [2].

This disorder is primarily caused by heterozygous mutations in ribosomal protein (RP) genes, leading to defective ribosome biogenesis and ribosomal RNA (rRNA) processing [3]. Recent studies using next-generation sequencing have identified additional RP and non-RP genes, such as *GATA1* [4], *TSR2* [5], and *HEATR3* [6]. RP gene mutations explain 50–60% of DBA cases, and mutations in non-RP genes account for less than 1% [3]. Approximately 20%–25% of DBA cases remain genetically unexplained [3]. The most commonly affected gene, *RPS19*, accounts for approximately 25% of DBA cases [7]. These findings have significantly expanded our understanding of the genetic heterogeneity and molecular pathophysiology of DBA, paving the way for more accurate diagnosis and potential new

therapeutic strategies. In this review, we focus on the genetic framework of DBA and offer insights into the associated mutations and their roles in the pathogenesis of the disease. The genes are listed in the order of their discovery.

RP genes and p53 pathway activation

Approximately 75% of DBA cases are caused by loss-of-function mutations in RP genes, typically affecting only one allele [3]. Ribosomes, which are composed of the 40S small subunit (containing RPS proteins) and the 60S large subunit (containing RPL proteins), are essential for translating mRNA into proteins, a process crucial for cell function and growth [8]. Mutations in *RPS* or *RPL* genes compromise functional ribosome production, resulting in defective rRNA processing and impaired subunit assembly [9,10]. *RPS* gene mutations in the 40S subunit disrupt 18S rRNA processing, leading to incomplete or nonfunctional small ribosomal subunits [10]. In the 60S subunit, *RPL* gene mutations impair the maturation of 28S and 5.8S rRNA, thus disrupting the assembly of the large subunit [9]. Although ribosomes are essential for all cell types, erythroid progenitor cells are particularly sensitive to defects in ribosome biogenesis [11]. This heightened sensitivity is due to the increased demand for protein synthesis during red blood cell (RBC) production [11]. Impaired ribosome production triggers a cellular stress response, particularly through the activation of the p53 pathway [11]. In DBA, RP mutations lead to the accumulation of free RPs that bind to and inhibit MDM2, a negative regulator of p53 [11]. This inhibition stabilizes and activates p53, resulting in increased apoptosis of erythroid progenitor cells in the BM [11].

40S Small Ribosomal Subunit (RPS genes)

RPS19 (Autosomal dominant [AD])

The *RPS19* gene is the first gene identified in relation to DBA and remains the most commonly mutated gene among affected individuals [12]. Draptchinskaia et al. [13] reported that in a female patient with DBA and a *de novo* balanced translocation t(X;19)(p21;q13), the *RPS19* gene was disrupted within its third intron. In a study screening for *RPS19* mutations in 40 unrelated individuals with DBA, Draptchinskaia et al. [13] identified nine distinct mutations in 10 patients. All individuals with mutations were heterozygous for these alterations, and no additional sequence variations were observed in the protein-coding region [13]. Willig et al. [14] examined 190 patients with DBA and discovered *RPS19* sequence alterations in approximately 24% of cases. Mutations in *RPS19* critically disrupt the maturation of the 3′ end of 18S rRNA, which is essential for the formation of the 40S ribosomal subunit [10]. This disruption leads to defects in ribosome assembly and ultimately activates the p53 pathway, resulting in apoptosis and impaired differentiation of erythroid progenitor cells [15].

RPS24 (AD)

Gazda et al. [16] conducted whole-exome sequencing (WES) and genetic linkage analysis on a cohort of patients with DBA and identified multiple mutations in *RPS24*, which interfered with its function and ultimately resulted in the failure of RBC production. This study emphasized the importance of ribosomal dysfunction in DBA, positioning *RPS24* alongside other RP genes involved in the disease [16]. The clinical features of patients with *RPS24* mutations typically include macrocytic anemia, with some cases presenting with congenital anomalies, such as growth retardation and craniofacial malformations [16]. Additionally, Choesmel et al. [17] demonstrated that *RPS24* mutations impair the maturation of 18S rRNA, a critical step in the formation of functional ribosomes, leading to defects in ribosome biogenesis and the disruption of erythropoiesis. Their experiments further revealed that *RPS24* mutations cause delayed pre-rRNA processing and failure to properly assemble the small ribosomal subunit [17]. Furthermore, this study highlights the functional interaction between *RPS19* and *RPS24*, suggesting that defects in multiple RPs may contribute to the disease's pathogenesis [17].

RPS17 (AD)

Cmejla et al. [18] discovered a *de novo* mutation in the *RPS17* gene in patients with DBA, macrocytic anemia, craniofacial dysmorphism, thumb/neck anomalies, congenital heart defects, urogenital malformations, and short stature [18]. Functional analyses have demonstrated that these mutations interfere with proper ribosomal assembly and prevent efficient translation, ultimately leading to the failure of RBC production [18]. The study further highlighted that a mutation in the *RPS17* gene, particularly affecting the start codon, significantly disrupts protein translation, thereby impacting ribosomal biogenesis [18].

RPS7 (AD)

Watkins-Chow et al. [19] demonstrated that *RPS7* mutations in mouse models caused developmental abnormalities, including reduced body size and neuroanatomical defects. Interestingly, unlike human *RPS7* mutations linked to anemia in

DBA, mouse models do not exhibit blood cell defects, highlighting potential species-specific differences in RP function [19]. Akram et al [20]. recently reported a novel *RPS7* variant that causes aberrant splicing, leading to DBA in mother and daughter. The mother experienced spontaneous remission of transfusion-dependent anemia at the age of 13 years, while her daughter was born with an occipital meningocele and later diagnosed with DBA [20]. This study expands the genotype-phenotype correlations in DBA, linking *RPS7* haploinsufficiency to hematologic abnormalities as well as neuroanatomical defects, such as meningocele [20].

RPS10 (AD)

Doherty et al. [21] identified three distinct mutations in *RPS10* in five patients: missense, nonsense, and frameshift mutations. These mutations affect the production of rRNA, particularly 18S rRNA, which is essential for the assembly of ribosomal small subunits [21]. Further analysis using pre-rRNA processing in patient-derived cells revealed that mutations in *RPS10* led to an abnormal accumulation of 18S-E pre-rRNA, impairing ribosomal biogenesis [21]. This is consistent with observations from knockdown studies in HeLa cells, in which reduced expression of *RPS10* caused similar defects in rRNA maturation, ultimately disrupting normal ribosomal function [21]. This study also used high-throughput sequencing to confirm that loss-of-function mutations in *RPS10* are major factors in DBA pathogenesis [22].

RPS26 (AD)

Doherty et al. [21] identified two unrelated DBA families with *RPS26* mutations, one affecting mRNA splicing and the other causing protein truncation. These mutations impair ribosome biogenesis by accumulating 18S-E pre-rRNA, leading to defective ribosome function and disrupted erythropoiesis, which contributes directly to the pathogenesis of DBA [21]. Gripp et al. [5] also reported two unrelated DBA families that carried *RPS26* mutations, one of which affected mRNA splicing, leading to improper gene expression, whereas the other caused protein truncation, disrupting ribosome biogenesis. These mutations are linked to defective ribosome assembly, a hallmark of DBA pathophysiology that impairs RBC production [5].

RPS29 (AD)

Mirabello et al. [23] identified two novel non-synonymous mutations in *RPS29* in two large families affected by DBA. These mutations result in the haploinsufficiency of *RPS29*, leading to defects in pre-rRNA processing and impaired ribosome biogenesis [23]. Functional studies using a zebrafish model showed that mutant *RPS29* failed to rescue defective erythropoiesis, confirming the pathogenic role of these mutations in DBA [23].

RPS28 (AD)

Gripp et al. [5] identified *RPS28* as a novel gene implicated in DBA and mandibulofacial dysostosis (MFD), including microtia or cleft palate, through WES. Two unrelated probands carried a *de novo* mutation affecting the start codon of the *RPS28* gene [5]. This mutation severely disrupts protein translation, leading to haploinsufficiency and impaired ribosome biogenesis [5]. This study also noted that experimental depletion of *RPS28* using siRNA resulted in reduced levels of other RPs, similar to the knockdown of *RPS19* [5].

RPS15A (AD)

Ikeda et al. [24] identified a splicing mutation in *RPS15A* (c.213G>A, p.K71K) in a DBA-affected family that caused aberrant splicing and production of a truncated transcript. The proband was diagnosed with DBA at 3 months of age, and BM study revealed severe erythroid hypoplasia (0%) with otherwise normal cellularity [24]. She had a total anomalous pulmonary venous connection and bilateral acetabular dysplasia [24]. There was a family history of anemia, as both her mother and older sister had experienced anemia during childhood, although there were no physical abnormalities [24]. The proband responded to corticosteroid therapy and eventually became steroid-independent [24]. Functional studies using CRISPR/Cas9 in human erythroid cells have shown that *RPS15A* haploinsufficiency disrupts 18S-E pre-rRNA processing and impairs 40S subunit assembly [24]. Zebrafish models further confirmed that *rps15a* knockdown leads to developmental defects, particularly impaired erythropoiesis, highlighting its role in DBA pathogenesis [24].

RPS27 (AD)

Wang et al. [25] identified loss-of-function mutations in the *RPS27* gene through WES in patients with DBA. A single nucleotide deletion in the *RPS27* gene led to a frameshift mutation, producing a premature stop codon, which causes defective ribosome biogenesis and impairs the pre-rRNA processing necessary for normal RBC production [25]. Functional studies using zebrafish models with *rps27* knockdown revealed devel-

opmental defects, including impaired erythropoiesis, further confirming the role of *RPS27* mutations in DBA pathogenesis [25].

60S Large Ribosomal Subunit (RPL genes)

RPL5 (AD)

Gazda et al. [26] found that *RPL5* mutations disrupt the maturation of pre-rRNA, particularly affecting the processing of 28S and 5.8S rRNA, which are essential for the assembly of the large ribosomal subunit. This disruption of ribosome biogenesis impairs erythropoiesis and contributes to the clinical manifestations of DBA [26]. *RPL5* mutations are frequently associated with distinct craniofacial and skeletal abnormalities, including cleft palate, thumb abnormalities, and congenital heart defects [26]. In a study of 196 patients with DBA, *RPL5* mutations were found in a subset of patients, many of whom exhibited these malformations, highlighting a strong genotype-phenotype correlation [26]. Furthermore, *RPL5* mutations tend to result in a more severe phenotype than mutations in other RP genes, such as *RPS19* [3]. Approximately 70% of patients with DBA with *RPL5* mutations present with congenital malformations, and cleft lip or palate is significantly more common in these patients than in those with other mutations [3].

RPL11 (AD)

Gazda et al. [26] have reported that individuals with *RPL11* mutations display a characteristic phenotype that includes both congenital abnormalities and DBA. Notably, *RPL11* mutations are predominantly associated with isolated thumb malformations, whereas *RPL5* mutations lead to a broader spectrum of physical abnormalities, including cleft palate and heart anomalies [26].

RPL35A (AD)

Farrar et al. [27] found that *RPL35A* is associated with DBA through high-resolution genomic mapping and gene expression microarray analyses. Researchers have identified *RPL35A* as a candidate gene in patients with DBA with chromosome 3q deletions and confirmed mutations in additional patients through sequence analysis [27]. Patients with *RPL35A* mutations exhibit both hematological and congenital features typical of DBA, including anemia in infancy and congenital anomalies affecting the craniofacial region, heart, genitourinary system, and upper limbs [27]. Some patients also display neutropenia, thrombocytopenia, and a heightened risk of developing hematological malignancies and osteosarcoma [27].

RPL26 (AD)

Gazda et al. [28] identified *RPL26* as a gene associated with DBA through largescale screening of RP genes in a cohort of 96 patients. Researchers have reported that a patient with an *RPL26* frameshift mutation exhibited DBA and multiple physical abnormalities, including craniofacial, upper limb, and cardiac malformations [28]. Interestingly, this mutation leads to defective ribosome biogenesis, affecting both small and large ribosomal subunits and disrupting the maturation of 18S and 28S rRNAs [28]. Additionally, the patient presented with macrocytic anemia and elevated erythrocyte adenosine deaminase levels [28].

RPL15 (AD)

Landowski et al. [29] identified a novel *RPL15* deletion in a patient with DBA that disrupted the formation of the 60S subunit and impaired pre-rRNA processing, particularly affecting the maturation of the 28S and 5.8S rRNAs. This leads to reduced ribosome production and impaired erythropoiesis [29]. Similarly, Wlodarski et al. [30] reported truncating mutations in *RPL15* in patients with severe hydrops fetalis, who later achieved spontaneous remission [30]. These mutations cause defects in ribosome assembly, resulting in decreased cell proliferation, delayed erythroid differentiation, and TP53-mediated apoptosis of hematopoietic cells [30]. Overall, *RPL15* mutations are linked not only to the development of DBA but also to unique clinical outcomes, such as spontaneous remission and treatment independence [30].

RPL27 (AD)

Using WES, Wang et al. [25] identified a splicing mutation in *RPL27* in a patient with DBA having an atrial septal defect and pulmonary stenosis, leading to defective ribosome biogenesis. Functional analysis using knockdown experiments in human erythroid cells revealed that depletion of *RPL27* impaired pre-rRNA processing, specifically affecting the maturation of 28S rRNA, which is essential for the proper formation of the 60S ribosomal subunit [25]. Moreover, zebrafish models with *RPL27* knockdown exhibited abnormal development, including reduced erythropoiesis, mimicking the anemia observed in patients with DBA [25].

RPL18 (AD)

Mirabello et al. [31] identified a non-synonymous *RPL18*

variant in two DBA-affected family members. One proband presented with anemia at 8 months of age and was steroid-responsive [31]. Another patient, the proband's child, exhibited mild anemia at birth, which later recurred at the age of one and also responded to steroid therapy [31]. None of the patients exhibited dysmorphic features, but both had intermittent neutropenia from birth [31]. This mutation causes the accumulation of 36S pre-rRNA, disrupts the maturation of 28S rRNA, and impairs the assembly of the 60S subunit, which hinders ribosome biogenesis and leads to defective erythropoiesis, contributing to the clinical symptoms of DBA [31].

RPL35 (AD)

Mirabello et al. [31] also identified a non-synonymous variant of *RPL35* in a family with DBA. The proband developed anemia at 2 months of age, which spontaneously resolved by 18 years of age without any known relapse [31]. BM evaluation at 3 months revealed erythroid hypoplasia, leading to the diagnosis of DBA [31]. The proband's daughter presented with anemia at 1 month of age, which was responsive to steroid therapy [31]. However, at age 15, she developed ulcerative colitis, and during treatment, her blood count dropped, leaving her dependent on RBC transfusions [31]. This mutation impairs pre-rRNA processing, leading to the accumulation of 32S pre-rRNA and defective 28S rRNA maturation, which disrupts the 60S subunit assembly and contributes to the clinical features of DBA [31].

Non-RP genes

GATA1 (X-linked recessive)

GATA1 plays a crucial role in regulating gene expression and the maturation of erythroid cells; in its absence, erythroid progenitors are unable to differentiate properly and ultimately undergo apoptosis [32]. Although important insights into *GATA1* function have been derived from animal models, the discovery that rare red cell disorders, such as DBA, are associated with *GATA1* mutations has provided a deeper understanding [32]. *GATA1* was recognized as the first non-RP mutation in DBA using WES [4]. A recent study in human cells revealed that the reduced translation of *GATA1* due to RP haploinsufficiency, a common cause of DBA, plays a key role in the erythroid abnormalities observed in this disorder [33,34]. Ludwig et al. demonstrated that *GATA1* mRNA has a high threshold for translation initiation, making it particularly susceptible to defects in RP levels [34]. In patients with DBA having *RPS19* mutations, despite unchanged *GATA1* mRNA levels, the activity of *GATA1* target genes is significantly diminished, indicating a translational defect [34]. The study also examined the effect of reducing *RPL11*, *RPL5*, and *RPS24* and found that these reductions similarly decreased GATA1 protein levels, suggesting a general mechanism [34]. This study offers strong evidence that impaired translation of *GATA1* mRNA, resulting from RP haploinsufficiency, plays a crucial role in the erythroid defects observed in DBA [33]. This aligns with the discovery that rare mutations in the *GATA1* gene itself can lead to disease, effectively linking the two mechanisms [33]. These insights could potentially be harnessed therapeutically, possibly by focusing on enhancing GATA1 protein production to alleviate anemia associated with DBA [33]. Additionally, Rio et al. [35] demonstrated that decreased HSP70 levels lead to a reduction in GATA1, causing an imbalance between globin and heme synthesis in DBA. This imbalance results in excess free heme, increased reactive oxygen species, and enhanced apoptosis of erythroid cells. The study shows that restoring HSP70 expression can rebalance globin and heme synthesis, reduce free heme toxicity, and improve erythropoiesis in DBA [35].

TSR2 (X-linked recessive)

Gripp et al. [5] explored the genetic basis of DBA combined with MFD in seven individuals from six unrelated families using WES of these individuals and their family members. They identified mutations in known DBA genes, such as *RPS26*, along with novel mutations in *TSR2* and *RPS28* [5]. Specifically, the *TSR2* hemizygous mutation has been analyzed for its effect on RP interactions and RNA processing, with researchers confirming that the mutation impairs the ability of the protein to bind to RPS26, a key step in ribosome assembly [5]. This study highlights the genetic heterogeneity of the combined DBA and MFD phenotypes, suggesting that disrupted ribosomal function may underlie the diverse clinical manifestations observed across different ribosomopathies [5]. A recent study by Yang and Karbstein [36] demonstrated that the chaperone TSR2 plays a crucial role in managing the release and reintegration of RPS26 from mature ribosomes, facilitating a reversible response to stress. Under stressful conditions, RPS26 dissociates from fully assembled ribosomes and triggers a targeted translational response [36]. TSR2 is essential for this process, aiding the release of RPS26 during stress and its reintegration into ribosomes once normal conditions are restored [36]. This mechanism enables ribosomes to swiftly adapt to environmental changes with minimal energy use, without compromising quality control [36]. Moreover, this study identified

a specific residue in RPS26 linked to DBA that influences the sodium stress response, highlighting the significance of this ribosome remodeling mechanism in ribosomopathies [36]. Together, these findings enhance our understanding of ribosomal heterogeneity and its role in stress responses and provide valuable insights into the development of DBA and related disorders [36].

HEATR3 (Autosomal recessive)

A recent study by O'Donohue et al. [6] showed that DBA can also be caused by biallelic mutations in the *HEATR3* gene. Six individuals from four families with biallelic mutations in *HEATR3* showed BM failure with selective erythroid hypoplasia, short stature, facial dysmorphism, limb deformities, cardiac defects, and intellectual disability [6]. *HEATR3* mutations destabilize a protein that is crucial for importing the RPs uL5 (RPL11) and uL18 (RPL5) into the nucleus, which are essential for ribosome assembly and p53 stabilization [6]. This study demonstrated that *HEATR3* mutations or reduced HEATR3 expression led to impaired cell growth, differentiation, and ribosome subunit formation, mimicking the effects of mutations in large subunit RP genes associated with DBA [6]. Furthermore, HEATR3-deficient cells exhibit decreased nuclear accumulation of RPL5 and abnormal erythrocyte maturation, independent of p53 activation [6]. Appropriate ribosome biogenesis is essential for the proliferation and differentiation of erythroid progenitors into RBCs [37]. In normal erythroid progenitors within the BM, HEATR3 functions as a transport factor moving RPL5 from the cytoplasm to the nucleus [37]. After entering the nucleus, RPL5 binds with RPL11 and 5S rRNA to form the 5S ribonucleoprotein complex, which is subsequently incorporated into the assembly of large ribosomal subunits, contributing to the formation of the central protuberance [37]. However, biallelic *HEATR3* mutations disrupt this process, leading to defects in pre-RNA processing, reduced 60S ribosomal subunits, and failure in erythropoiesis, which clinically manifests as DBA [37].

CONLCLUSION

In summary, this review highlights significant advances in understanding the genetic underpinnings of DBA, particularly the role of mutations in RP genes. These findings underscore the critical role of ribosomal dysfunction in DBA pathogenesis, which contributes to defective ribosomal biogenesis, p53 pathway activation, and impaired erythropoiesis. In addition, the identification of non-RP gene mutations broadens the genetic landscape of DBA and suggests that ribosomal stress and erythroid-specific defects may arise from a wider array of genetic abnormalities. This reinforces the complexity of DBA as a ribosomopathy and highlights the need for further exploration of non-RP gene mutations.

ACKNOWLEDGEMENT

None.

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JIG Journal of Interdisciplinary Genomics

https://doi.org/10.22742/JIG.2024.6.2.33
Journal of Interdisciplinary Genomics 2024;6(2):33-36
eISSN 2671-6771

Clinical Application of Chromosomal Microarray for Hematologic Malignancies

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Chromosomal microarray (CMA) can detect genome-wide small copy number abnormalities (CNAs) and copy-neutral loss of heterozygosity (CN-LOH) better than conventional karyotyping and fluorescence in situ hybridization (FISH) for hematologic malignancies. Apart from the limitations in detecting balanced chromosomal rearrangements and low-level malignant clones, CMA has clinical utility in detecting significant recurrent and novel variants with diagnostic, prognostic, and therapeutic evidence. It can successfully complement conventional cytogenetic tests for several hematological malignancies, including acute myeloid leukemia (AML), myelodysplastic syndrome (MDS), acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), and multiple myeloma (MM). An increase in CMA testing for hematologic malignancies is expected to identify novel markers of clinical significance.

Key words: Chromosomal microarray, Hematologic malignancy, Copy-number abnormalities, Copy-neutral loss of heterozygosity

REVIEW ARTICLE

Received: October 2, 2024
Revised: October 22, 2024
Accepted: October 23, 2024

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INTRODUCTION

Conventional karyotyping is the primary test used to detect chromosomal aberrations in hematological malignancies. This is useful for detecting numerical aberrations and balanced rearrangements [1]. However, karyotyping has limitations, such as low resolution, lack of objective parameters to define G-banding patterns, and dependence on cell culture efficiency. Fluorescence in situ hybridization (FISH) can overcome the limitations of karyotyping. However, FISH can be used only for specific chromosomal regions [2].

Chromosomal microarray (CMA) is widely used to detect small copy number variants (CNVs) and is primarily recommended for application in germline disorders such as neurodevelopmental disorders and congenital malformations [3]. CMA can detect copy-neutral loss of heterozygosity (CN-LOH) using single nucleotide polymorphism (SNP) markers, and is applicable for diagnosing imprinting disorders [4]. CMA is also applicable to the genetic diagnosis of somatic disorders such as hematologic malignancies and lymphomas [5,6]. In this article, we describe the clinical utility of CMA in the genetic investigation of hematologic malignancies.

BENEFITS AND LIMITATIONS OF CMA FOR HEMATOLOGIC MALIGNANCIES

Recent CMA platforms consist of CNV and SNP markers. The CNV compares the scanned data of the sample with the control data, which were obtained using hundreds of control individuals [7]. Therefore, the application of a control sam-

ple is not required, which is required for conventional array comparative genomic hybridization (aCGH) [8]. SNP markers are complementary to CNV markers and help detect CNVs more accurately using B-allele frequencies [9]. A combination of CNV and SNP markers can be used to distinguish between heterozygous deletions and CN-LOH [10].

The application of CMA to hematological malignancies does not require cell culture processing, which is crucial for karyotyping and FISH. Therefore, CMA can avoid cell culture bias and may have a shorter turnaround time than karyotyping [10]. Most CMAs have a higher resolution than karyotyping and are much more sensitive for detecting small copy number abnormalities (CNAs) with sizes < 5–10 Mb [11]. It can also discern complex chromosomal abnormalities such as amplification, chromothripsis, intrachromosomal complexity, and genomic complexity [12]. The distinction between the doubling of hypodiploid clones of acute lymphoblastic leukemia (ALL) and non-hypodiploid ALL can be achieved by CMAs [10].

However, CMA has certain limitations. Generally, balanced rearrangements cannot be detected, such as balanced translocations or inversions. The CMA results depend on the proportion of malignant cells in the sample, and has limitations in detecting minimal residual diseases (Table 1). This is because CMA cannot detect low levels of mosaicism or chimerism with a percentage < 20% [13]. CMA cannot distinguish between individual clones, such as stemlines and sidelines either [14]. CMA is not recommended for all types of hematologic malignancies [15]. The interpretation of CMA results can be difficult for hematologic malignancies compared to germline disorders because the public database is limited to somatic CNAs [12].

INDICATIONS OF HEMATOLOGIC MALIGNANCIES FOR CMA

All hematological malignancies were not indicated in the CMA analysis. Generally, the diagnostic and prognostic benefits of CMA are limited to chronic myelogenous leukemia (CML) and myeloproliferative neoplasms (MPN) [15]. However, CMA can sensitively detect recurrent or novel findings in acute myeloid leukemia (AML), myelodysplastic syndrome

Table 1. Comparison of advantages and disadvantages in cytogenetic tests for hematologic malignancies

Method	Advantages	Disadvantages
Karyotyping	- Direct observation of all chromosomal abnormalities - Can detect balanced translocations	- Low resolution - Requires cell culture - Cannot detect small CNAs
FISH	- High sensitivity for specific chromosomal abnormalities	- Limited to predefined regions - Requires pre-designed probes
CMA	- High-resolution detection of CNAs - No need for cell culture - Can detect CN-LOH	- Cannot detect balanced translocations - Depends on the proportion of malignant cells - Limited ability to detect low-level mosaicism

FISH, fluorescence in situ hybridization; CMA, chromosomal microarray; CNA, copy number abnormality; CN-LOH, copy neutral loss of heterozygosity.

Table 2. Indications and suggestive findings of hematologic malignancies for chromosomal microarray

Indication	Suggestive findings
AML	-5/5q del, -7, KMT2A partial tandem dup, 13q CN-LOH, 9q del
MDS	-5/5q del, -7/7q del, Trisomy 8, 11q del, 12p del, -13/13q del, 17p del/i(17q), 7q CN-LOH, 11q CN-LOH, 1p CN-LOH, 1q gain, Trisomy 21
Myeloid/lymphoid neoplasms with eosinophilia	4q12 del (FIP1L1-PDGFRA fusion)
B-ALL	-5/5q del, -7/7q del, Trisomy 8, 11q del, 12p del, -13/13q del, 17p del/i(17q), IKZF1 del (7p12.2), ERG del (21q22.2), CDKN2A/2B del (9p21.3), ETV6 del (12p13.2), PAX5 del (9p13.2), RB1 del (13q14.2)
T-ALL	TCR rearrangements with CNAs, 9q34.1 amp in NUP214-ABL1 fusion, 1p33 del in STIL-TAL1 fusion, 6q del, CDKN2A/2B biallelic del (9p21.3)
CLL	11q22.3 del (ATM and/or BIRC3), Trisomy 12, 13q14.2 del (MIR15A/16-1), 17p13.1 del (TP53), 2p12p25.3 gain (MYCN), 9p21.3 del (CDKN2A), Trisomy 19, 6q del, 14q24.1q32.3 del
MM	Trisomies of odd-numbered chromosomes, 1q21 gain, -17/17p13.1 del (TP53), 1p del, 14q del, 16q del
Burkitt-like lymphoma with 11q aberrations	11q CNAs

AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; ALL, acute lymphoblastic leukemia/lymphoma; CLL, chronic lymphocytic leukemia; MM, multiple myeloma; del, deletion; dup, duplication; amp, amplification; CN-LOH, copy-neutral loss of heterozygosity; CNA, copy number abnormality.

(MDS), ALL, chronic lymphocytic leukemia (CLL), and multiple myeloma (MM) [11,16-21]. CMA application is recommended as the next step to detect novel findings such as small CNAs and CN-LOH, if normal results are obtained at the diagnosis or relapse of hematologic malignancies through karyotyping and FISH, CMA testing is also recommended as an alternative if the cell culture for karyotyping fails [10]. In ALL, some CNAs are indicative of gene fusions, such as 1p33 deletion (STIL-TAL1 fusion) and 9q34.1 amplification (NUP214-ABL1 fusion), which have diagnostic values (Table 2) [10,12].

LABORATORY STANDARDS AND QUALITY ASSURANCE OF CHROMOSOMAL MICROARRAY FOR HEMATOLOGIC MALIGNANCIES

Validation or verification of testing is required in the laboratory before clinical practice of CMA testing is conducted . During the validation process, the accuracy, precision, analytical sensitivity, specificity, and reportable range must be established. During the verification process applicable to Food and Drug Administration-approved tests, the accuracy, precision, and reportable range of results must be established using previously characterized samples. The percentage of abnormal cells was determined by a dilution study using samples with known copy number changes [13].

The laboratory must establish sample requirements and DNA quality thresholds. Generally, the primary recommended sample for hematologic malignancy is bone marrow (BM). A peripheral blood (PB) sample can be used as an alternative if malignant cells are sufficient in the PB [15]. The laboratory must establish thresholds for quality control (QC) metrics in assay procedures, such as DNA OD 260/280, quantity, and PCR product size requirements. The thresholds of data QC metrics, such as the median absolute pairwise difference (MAPD) and SNPQC, must be established and managed in the laboratory [7,13].

INTERPRETATION OF CMA RESULTS FOR HEMATOLOGIC MALIGNANCIES

The American College of Medical Genetics and Genomics (ACMG) and the Cancer Genomics Consortium (CGC) reported consensus recommendations regarding technical standards for the interpretation of CNAs and CN-LOH in neoplastic disorders. Interpretation of the CMA results for hematologic malignancies was based on a four-tier evidence-based categorization system. This system is similar to the sequence variant interpretation standards for somatic disorders and focused on the diagnostic, prognostic, and therapeutic significance. According to the evidence level, the CNAs or CN-LOH of the CMA results can be classified as Tier 1A/B (strong clinical significance), Tier 2 (some clinical significance), Tier 3 (clonal variants with no documented association with neoplastic disorder), and Tier 4 (benign or likely benign). Under special considerations, the germline pathogenic variants associated with cancer predisposition are classified as Tier 1A [12].

The interpretation of CMA results for hematologic malignancies can be highly dependent on other clinical information, such as clinical/pathologic diagnosis, and other test results, including karyotyping, FISH, and other molecular analyses. The same cytogenomic aberrations can be classified differently in different disorders.

Several public databases contain information on somatic copy number abnormalities. There is a lack of public data, except for the World Health Organization classification of hematolymphoid tumors. The laboratories are recommended to manage in-house databases to discriminate between significant and normal results. It is also recommended that laboratory standards be established to report incidental findings such as suspected germline variants associated with other clinical relevance such as constitutional disorders [12,22].

SUMMARY AND CONCLUSION

CMA is widely used in the diagnosis of hematologic malignancies such as AML, MDS, ALL, CLL, and MM. Although CMA has limitations in detecting balanced chromosomal rearrangements, it exhibits diagnostic utility for detecting small CNAs and CN-LOH. The CMA results for hematologic malignancies are clinically significant as diagnostic, prognostic, and therapeutic evidence. In Korea, healthcare reimbursements are necessary for the clinical application of CMA for hematologic malignancies. CMA testing is highly recommended to complement conventional karyotyping and FISH in various hematologic malignancies. An increase in CMA testing for hematologic malignancies is expected to provide novel diagnostic and prognostic findings for optimizing patient care and treatment.

CONFLICT OF INTEREST

I declare that I do not have any conflicts of interests.

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JIG Journal of Interdisciplinary Genomics

https://doi.org/10.22742/JIG.2024.6.2.37
Journal of Interdisciplinary Genomics 2024;6(2):37-41
eISSN 2671-6771

Neonatal Diabetes Mellitus: A Focused Review on Beta Cell Function Abnormalities

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Neonatal diabetes mellitus, or congenital diabetes mellitus, is a rare genetic disorder caused by abnormal β cell function and other causes. The symptoms of hyperglycemia that occur in neonatal diabetes. The symptoms of hyperglycemia that occur in neonatal diabetes may be transient or persistent. The most frequent genetic cause of neonatal diabetes characterized by abnormal β cell function is abnormalities at the 6q24 locus. Another possible cause is mutations in the *ABCC8* or *KCNJ11* genes, which code for potassium channels in pancreatic β cells. This underscores the importance of rapid genetic diagnosis following neonatal diabetes diagnosis and highlights the critical timing of sulfonylurea use.

Key words: Neonatal diabetes mellitus, Gene, 6q24, K_{APT} channels

REVIEW ARTICLE

Received: October 15, 2024
Revised: October 20, 2024
Accepted: October 21, 2024

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INTRODUCTION

Neonatal diabetes mellitus, also known as congenital diabetes mellitus, is a rare genetic disorder with an incidence of approximately 1 in 10,000 live births [1,2]. Neonatal diabetes is defined as diabetes diagnosed within the first six months of life [3]. Although diabetes generally arises from complex interactions between environmental and genetic factors, neonatal diabetes arises specifically from genetic defects [2]. Neonatal diabetes is primarily associated with abnormalities in the development and secretion of insulin-producing cells in the pancreas, or with dysfunction of pancreatic β cells. The most frequent genetic causes of neonatal diabetes characterized by abnormal β-cell function include abnormalities at the 6q24 locus and mutations in the *ABCC8* or *KCNJ11* genes, which code for potassium channels in pancreatic β cells [1,2].

Other genetic anomalies have been reported, which are associated with pancreatic development, abnormalities in β cell differentiation, and apoptosis [4]. Neonatal diabetes is classified based on insulin dependency into the transient (temporary) and permanent [3] forms. In the transient form, treatment can be discontinued at any time from the first few weeks up to age five years, whereas the permanent form requires lifelong treatment. The clinical differences between transient and permanent neonatal diabetes are not always associated with distinct molecular mechanisms. Abnormalities at the 6q24 locus are solely associated with transient neonatal diabetes, whereas mutations in *ABCC8*, *KCNJ11*, and *INS* are linked to both permanent and transient forms [5-7]. Other genetic factors are also associated with permanent neonatal diabetes [4]. At birth, 62% of neonates have a birth weight below the 10th percentile, underscoring the critical role of insulin secretion in fetal growth [3]. In patients with transient diabetes, the condition recurs at the onset of puberty in 86% of cases, likely due to insulin resistance

associated with puberty [8]. No differences were observed among the genetic groups, and neonatal diabetes can be accompanied by neurological disorders and developmental defects [9]. Genetic analysis can diagnose monogenic diabetes in nearly 83% of patients before six months of age [10]. Genetic diagnosis is essential as it influences treatment options and can predict potential diabetes-related complications or illnesses. Genetic analysis should be performed for all children in the following cases: those diagnosed with diabetes within six months of birth; those aged six months to 1 year who exhibit extra-pancreatic features and lack evidence of pancreatic autoimmunity; and those with multiple autoimmune disorders, unusual family history, and associated congenital disabilities. Testing should not be delayed until other symptoms or potential remission [11]. Furthermore, it is crucial to ascertain whether sulfonylureas can be successfully introduced [12].

GENETIC ASPECTS (ABNORMAL β CELL FUNCTION)

Abnormalities at the 6q24 locus (*PLAGL1* and *HYMAI*)

Neonatal diabetes resulting from abnormalities in the 6q24 locus (MIM#601410 and 603044) encompasses paternal uniparental disomy of 6q24 (pUPD6), partial duplication of paternal 6q24, and relaxation of the maternal 6q24 imprinted locus [13]. This locus contains a CpG island with differential methylation depending on parental origin. Methylation downregulates the gene transcription of methylated alleles [13]. Overexpression of imprinted genes located at 6q24, such as *PLAGL1* (pleiomorphic adenoma gene-like 1) and *HYMAI* (hydatidiform mole-associated and imprinted) transcript, is believed to be associated with disease manifestations [14,15] (Table 1). *PLAGL1* is a transcription factor that encodes a protein involved in the regulation of cell cycle arrest and apoptosis, as well as in the induction of the receptor one gene for the potent insulin secretagogue human pituitary adenylate cyclase-activating polypeptide (PACAP1). Diabetes resulting from abnormalities at the 6q24 locus typically occurs before one month of age in 93% of cases, and before three months in 100% of cases. Intrauterine growth restriction was observed across all genetic groups, with a higher percentage found in patients with 6q24 abnormalities than in those with *ABCC8* or *KCNJ11* mutations. Reports indicate that 97% of patients with 6q24 locus abnormalities experience remission before one year of age [16]. Additionally, patients with 6q24 locus abnormalities can experience developmental defects (such as macroglossia, umbilical hernia, cardiac malformations, renal and urinary malformations, non-autoimmune anemia, and hypothyroidism with glands in situ) and neurological disorders [16].

Mutations of the K_{ATP} Channel Genes (*ABCC8* and *KCNJ11*)

The ATP-sensitive potassium channel (K_{ATP} channel) (MIM *600509 and *600937) is critical in stimulating insulin secretion in response to glucose in pancreatic β cells. Under low blood glucose conditions, K_{ATP} channels are activated and remain open, maintaining a hyperpolarized resting membrane potential. When blood glucose levels rise, glucose is taken up into β cells, entering the glycolytic pathway and increasing intracellular ATP concentration. This leads to the closure of K_{ATP} channels, resulting in potassium accumulation within the cell, ultimately causing membrane depolarization. Depolarization activates voltage-dependent calcium channels, allowing Ca^{2+} ions to enter β cells, facilitating the exocytosis of secretory vesicles, and releasing insulin into the bloodstream. ATP channels comprise a tetrameric protein structure formed by two subunits encoded by *KCNJ11* and *ABCC8* [17,18]. Even in individuals with a normally structured pancreas, activation mutations in either of these genes can disrupt the structure or function of K_{ATP} channels, leading to neonatal diabetes. These mutations cause the K_{ATP} channel to remain permanently open, thereby failing to regulate membrane potential in response to rising glucose levels, which ultimately results in impaired insulin secretion. Mutations in the *ABCC8* and *KCNJ11* genes man-

Table 1. Genetic causes of neonatal diabetes mellitus

Abnormality point	Gene	Gene function	Tansmission mode	Type of diabetes
6q24 locus methylation	*PLAGL1, HYMAI*	Transcription factor regulation of cell cycle arrest and apoptosis	Genetic aberrations of the imprinted locus at 6q24	Mostly transient, rare permanent
K_{ATP} Channel	*ABCC8, KCNJ11*	K_{ATP} channel/insulin secretion	AD	Permanent, transient, DEND
Pro-insulin	*INS*	Hormone	Rare AR	Transient, permanent
Glucokinase	*GCK*	Glucose metabolism	AD, AR	Heterozygous: MODY2 Homozygous: permanent

AD, autosomal dominant; AR, autosomal resessive; DEND, Developmental delay Epilepsy and Neonaral diabetes.

ifest in approximately 30% of cases before one month of age and between 1 and 6 months of age in 66% of cases [3]. In patients with *ABCC8* or *KCNJ11* mutations, remission may persist until five years of age [3,19]. Approximately 25% of these patients experience neurological disorders ranging from severe epilepsy to cognitive developmental delays, commonly referred to as DEND syndrome (developmental delay, Epilepsy, and Neonatal Diabetes) [20] (Table 1). Furthermore, when patients undergo detailed neuro-psychomotor and neuropsychological assessments, attention deficits or language disorders, including dyslexia, are observed in 100% of cases [3].

Mutations of the Insulin Gene (*INS*)

Mutations in the insulin gene (*INS*) (MIM *176730) predominantly involve heterozygous mutations affecting the structure of pro-insulin and are inherited in an autosomal dominant manner [21,22]. Pro-insulin with structural abnormalities is degraded within the cell, leading to severe endoplasmic reticulum (ER) stress and eventual β cell death. This process has been documented in mouse models and human studies, and reports suggest that INS mutations may influence β cell growth and development through chronic ER stress rather than solely leading to cell death [23-26]. Some mutations alter protein expression and are primarily inherited in a recessive manner within consanguineous families. These mutations have been shown to affect the expression of the insulin promoter directly or are influenced by mutations in factors that enhance its activity [27] (Table 1). Rare recessive *INS* mutations may lead to remission at a median age of 12 weeks. However, most *INS* mutations are dominant and do not lead to remission.

Mutations of the *Glucokinase* Gene (*GCK*)

Glucokinase plays a pivotal role in the first step of glucose metabolism in β cells and acts as a glucose sensor to regulate the amount of insulin secreted. Nonsense mutations of the *GCK* gene (MIM *138079) lead to Maturity Onset Diabetes of the Young type 2 (MODY 2), which typically presents as moderate hyperglycemia [28]. This genetic disorder is transmitted in an autosomal dominant manner; however, homozygous states of these nonsense mutations can result in a complete deficiency of glucokinase-mediated glycolysis, leading to neonatal diabetes [29] (Table 1). Although this is not a frequent cause of neonatal diabetes, it is crucial to check fasting blood glucose levels in both parents, mainly if there is a history of gestational diabetes. If mild glucose intolerance is found in both parents, evaluating mutations in the *GCK* gene is necessary.

THERAPEUTIC ASPECTS

Patients with neonatal diabetes often begin treatment during the neonatal period because of early-onset diabetes and intrauterine growth retardation. The initial treatment aims to rebalance carbohydrate metabolism and begins immediately upon diagnosis. Treatment should establish a balance between calorie and carbohydrate intake necessary to restore average weight (15–18 g/kg/day of carbohydrates) while avoiding excessive intake that could lead to future insulin resistance. Additionally, sufficient insulin-based treatment is required to achieve appropriate metabolic equilibrium. The goal is to normalize blood glucose levels without inducing hypoglycemia by targeting blood glucose levels before (target: 70–120 mg/dL) and after meals (target: 100–145 mg/dL). Both hyperglycemia and hypoglycemia can adversely affect the neurological development of neonates; therefore, it may be beneficial to use diluted insulin or an insulin pump to improve insulin management during the early weeks of life. Blood glucose measurements should accurately reflect capillary blood glucose levels and continuous glucose monitoring sensors may serve as alternatives.

Patients with mutations in the *ABCC8* or *KCNJ11* genes were successfully treated with hypoglycemic sulfonylureas. These sulfonylureas bind to the SUR1 subunit, which regulates potassium channels. In patients with these mutations, the K_{ATP} channels remain sensitive to sulfonylureas in approximately 90% of cases, inhibiting the potassium channels in pancreatic β cells and restoring insulin secretion in response to meals. Sulfonylurea therapy is reportedly safe and effective for controlling blood glucose levels in neonatal patients with diabetes, even before genetic test results are available. Therefore, empirical inpatient trials on sulfonylureas should be considered. Current evidence indicates that treatment with sulfonylureas normalizes HbA1c and significantly reduces the incidence of hypoglycemia while providing better metabolic control than insulin in neonatal diabetes associated with *ABCC8* or *KCNJ11* mutations. Recent studies have also demonstrated that when introduced early in childhood, hypoglycemic sulfonylureas can improve neurological, neuropsychological, and visuomotor impairments [33,34]. Moreover, Garcin et al. showed that sulfonylureas could successfully replace insulin in neonatal diabetes associated with chromosome 6 methylation abnormalities [35]. This underscores the importance of rapid genetic di-

agnosis following the diagnosis of neonatal diabetes and highlights the critical timing for the introduction of sulfonylureas.

CONCLUSION

Neonatal diabetes is a model for rare human genetic disorders and is pivotal for understanding beta cell function abnormalities, including issues related to the 6q24 locus and mutations in genes for the K_{APT} channel, pro-insulin, and glucokinase. Neonatal diabetes is often associated with specific neuropsychological or developmental disorders, necessitating all clinicians treating patients with neonatal diabetes to investigate the occurrence of these clinical symptoms. The treatment options for neonatal diabetes include insulin or sulfonylureas; the use of sulfonylureas is associated with a lower risk of hypoglycemia. Ultimately, it is essential to establish a prompt genetic diagnosis and prioritize the early introduction of sulfonylureas for the management of neonatal diabetes.

CONFLICT OF INTEREST

Not applicable.

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JIG Journal of Interdisciplinary Genomics

https://doi.org/10.22742/JIG.2024.6.2.42
Journal of Interdisciplinary Genomics 2024;6(2):42-46
eISSN 2671-6771

Inhibition of STAT Transcription Factor Attenuates MPP$^+$-induced Neurotoxicity

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Background: The most prominent pathological features of Parkinson's disease (PD) are diminished substantia nigra (SN), which is part of the output component of the basal ganglia, the severe death of dopaminergic neuronal cell and the accumulation of a synuclein (αSYN). However, the mechanism by which αSYN causes toxicity and contributes to neuronal death remains unclear.
Methods: The aim of this study was to investigate the effect of αSYN/STAT oligodeoxynucleotide (ODN), which simultaneously suppresses STAT transcription factors and αSYN mRNA expression in an *in vitro* Parkinson's disease model.
Results: Synthetic αSYN/STAT ODN effectively inhibits 1-Methyl-4-phenylpyridinium (MPP$^+$) induced STAT phosphorylation and αSYN expression. αSYN/STAT ODN attenuated MPP$^+$ to mimic PD model *in vitro*. MPP$^+$ induced the secretion of TNF-α/IL-6, inhibited cell viability and induced apoptosis while these effects could be rescued by αSYN/STAT ODN.
Conclusion: Therefore, synthetic αSYN/STAT ODN has substantial therapeutic feasibility for the treatment of neurodegenerative diseases.

Key words: Parkinson's disease, Oligodeoxynucleotide, α-Synuclein, Apoptosis, STAT

ORIGINAL ARTICLE

Received: September 30, 2024
Accepted: October 21, 2024

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INTRODUCTION

Parkinson's disease (PD) is a chronic, widespread neurodegenerative disorder characterized by a progressive loss of dopaminergic neurons in the substantia nigra (SN) pars compacta (SNpc) throughout the midbrain [1]. The main pathology of PD is the aggregation of the protein α-synuclein (αSYN) in the cytoplasmic region of dopamine neurons [2].

The Janus Kinase/Signal Transducers and Activators of Transcription (JAK/STAT) is activated by cytokines, interferons, and growth factors [3] and is involved in cell survival, proliferation, angiogenesis, inflammation, and apoptosis [4]. Abnormal activation of JAK/STAT occurs in neuroinflammation and neurodegenerative diseases such as multiple sclerosis, Alzheimer's disease, and PD [5].

Synthetic oligodeoxynucleotide (ODN) technology is a gene therapy strategy consisting of DNA or RNA-based molecular compounds that disrupt gene transcription or translation [6]. To improve a new therapeutic approach, in this study we used a combination of antisense ODN and decoy ODN to synthesize αSYN/STAT ODN which inhibits both SYN and STAT. Although these ODN have proven beneficial in several disease models, it has not yet been demonstrated whether αSYN/STAT ODN can attenuate the development of molecular mechanisms of neurotoxicity. Therefore, we investigated the effect of αSYN/STAT ODN on neuronal cytotoxicity in an *in vitro* model of Parkinson's disease.

METHODS

Synthesis of Oligodeoxynucleotides (ODN)

Synthetic ODNs were commissioned by Macrogen (Seoul, Korea). The synthetic decoy ODN sequences were used as follows (the target site of the consensus sequence is underlined): STAT decoy ODN: 5′ GAA TTC GTT TCC GGG AAT GAA AAC ATT CCC GGA AAC 3′; αSYN antisense ODN: 5′ GGT ACC CTT CTT CAC CCT TAC C 3′; scrambled (SCR) decoy ODN: 5′ GAA TTC AAT TCA GGG TAC GGC AAA AAA TTG CCG TAC CCT GAA TT 3′. Considering the stability of the decoy ODN strategy, we designed a ring-type structured decoy ODN. These ODNs were annealed for 6 hours while temperature was gradually decreased from 80°C to 25°C. Each ODN was mixed with T4 ligase (Takara Bio, Otsu, Japan) and incubated for 18 hours at 16°C to obtain a covalent ligation for the ring-type decoy ODNs.

Cell culture and Reagents

A dopaminergic human neuroblastoma cell line SH-SY5Y (America Tissue Culture Collection, CRL-2266; ATCC, Manassas, VA, USA), was cultured in a Dulbecco's Modi-fied Eagle's Medium (DMEM) medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Gibco) and 1% Anti-Anti (Gibco). Cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2. The sources of the following reagents were: 1-Methyl-4-phenylpyridinium ion (MPP^+) (Sigma-Aldrich); anti-SYN (Cat no: 2628, Cell Signaling Technology), anti-PARP-1 (Cat no: 9542, Cell Signaling Technology), anti-pSTAT3 (Cat no: 9145, Cell Signaling Technology) and anti-β-actin (Cat no: SAB3500350, Sigma-Aldrich). Immunoblots were detected using an enhanced chemiluminescence reagent (Amer-sham Bioscience, Amersham, UK).

Cytotoxicity assay

To evaluate the effect of αSYN/STAT ODN on MPP^+ stimulated proliferation SH-SY5Y cells were plated in 96-well culture plates at 1×10^5 cells/ml in culture medium and allowed to attach for 24 hours. Media were discarded and transfect with αSYN/STAT ODN in a new medium, then treat with MPP^+ for 24 hours. Cell viability was analyzed using the Cell Counting Kit (CCK-8; Dojindo Laboratories, Kumamoto, Japan) assay according to the manufacturer's instructions. The absorbance at 450 nm was assessed using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

Transfection and Morphology examination

SH-SY5Y cells were transfected with synthetic ODN using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After transfection, the SH-SY5Y cells were cultured in MPP^+ for 24 hours. The morphology of SH-SY5Y cells were observed using an inverted phase contrast microscope (Olympus CKX41SF, Tokyo, Japan, ×200 magnification).

Immunoblot analysis

SH-SY5Y cells with a protein extraction buffer (N-PER™, Thermo Fisher Scientific, Waltham, MA, USA) according to the instruction manual. The protein samples were separated on precast gradient polyacrylamide gels (Bolt™ 4–12% Bis-Tris Plus Gels; Thermo Fisher Scientific) and transferred to nitrocellulose membranes (GE Healthcare, Madison, WI, USA) by using Bolt™ Mini Blot Module and Mini Gel Tank (Thermo Fisher Scientific), according to the manufacturer's recommendations. The membrane blocked with 5% bovine serum albumin was probed with a primary antibody and horseradish peroxidase-conjugated secondary antibody. Following a repeat of the wash step, the membrane was kept in enhanced chemiluminescence detection reagents (Thermo Fisher Scientific). Signal intensity was measured with an image analyzer (Chemi-Doc™ XRS+; Bio-Rad Laboratories).

Enzyme-linked immunosorbent assay (ELISA)

The culture medium of the cells was harvested, and cytokine production (TNFα and IL6) in the supernatant was measured with a solid phase sandwich ELISA using a Quantikine TNFα and IL6 kit (R&D systems, MN, USA) according to the manufacturer's instructions.

Statistical analysis

All data analysis was performed with the GraphPad Prism 9 (GraphPad Software, Inc., San Diego, CA, USA) using either a one-way ANOVA with Tukey's post hoc test for multiple comparisons and data are presented as the mean ± SEM (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

RESULTS

αSYN/STAT ODN protects SH-SY5Y cells against MPP^+ induced neurotoxicity

The cytotoxic effects of αSYN/STAT ODN on SH-SY5Y cells were examined through a CCK assay before investigating its

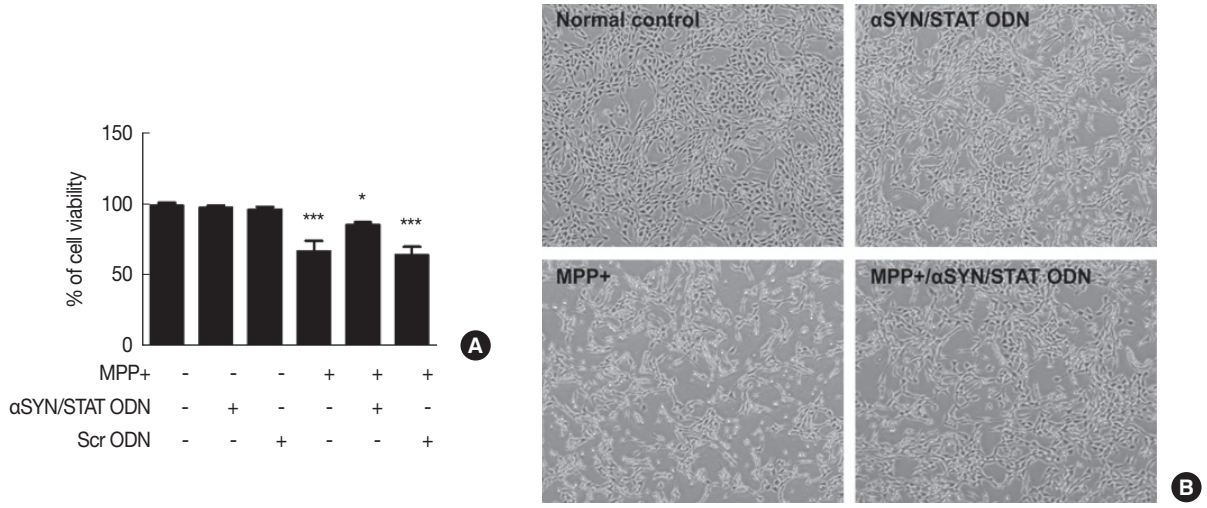

Fig. 1. Effect of αSYN/STAT oligodeoxynucleotide (ODN) on MPP+ to mimic Parkinson's disease (PD) model *in vitro*. (A) Viability was determined using the MTT assay. (B) The morphological changes, magnifications ×200. The data are representative of three similar experiments and quantified as mean values±SEM. *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared to normal control.

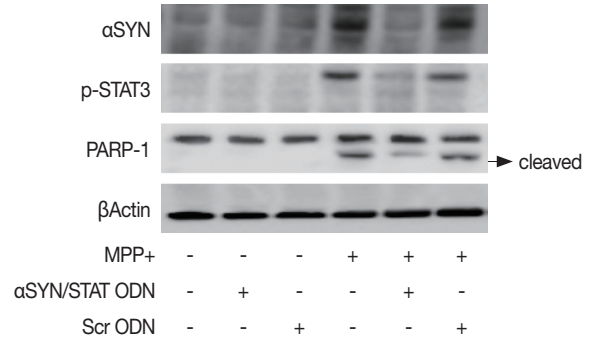

Fig. 2. Effect of αSYN/STAT oligodeoxynucleotide (ODN) on MPP+ induced SYH accumulation, STAT3 phosphorylation and cleaved PARP-1. Beta-actin was used to confirm equal sample loading.

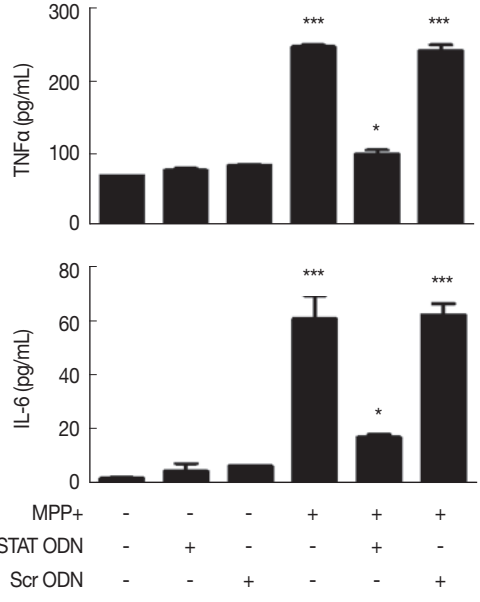

Fig. 3. Effect of αSYN/STAT oligodeoxynucleotide (ODN) on MPP+ induced neuroinflammatory responses. The data are representative of three similar experiments and quantified as mean values± SEM. *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared to normal control.

pharmacological potential. αSYN/STAT ODN significantly increased the viability of 3 mM MPP+ stimulated SH-SY5Y cells compared to cells treated with only MPP+ (Fig. 1A). Transfection of Scr ODN, a negative control, was similar to cells treated with MPP+. These results were also observed in cell morphology. SH-SY5Y cells grew well, showing obvious neurites, and the cells treated with only αSYN/STAT ODN did not show any difference in cell growth compared to normal cells (Fig. 1B). When SH-SY5Y cells were exposed to MPP+ or Scr ODN, neurites were reduced and cell debris increased; however, they were recovered with αSYN/STAT ODN transfection.

Effect of αSYN/STAT ODN on MPP+ induced apoptosis signaling pathway

Since apoptosis is one of the important steps in the pathogenesis of PD, we hypothesized that αSYN/STAT ODN could protect dopaminergic neuronal cells by inhibiting the apoptotic pathway. First, we confirmed changes in the expression of ODN target proteins caused by MPP+. As shown Fig. 2, expression of SYN and p-STAT3 were increased by MPP+ or Scr ODN. As expected, this increase was reduced by αSYN/STAT ODN. In addition, αSYN/STAT ODN inhibited MPP+ induced cleaved PARP-1, apoptosis marker protein, in SH-SY5Y cells.

αSYN/STAT ODN alleviates MPP+ induced neuroinflammatory response

MPP+ causes mitochondrial dysfunction and neuroinflammation [7]. Repression of the JAK/STAT pathway disrupts the neuroinflammation and neurodegeneration circuitry characteristic of PD [8]. To evaluate the impact of αSYN/STAT ODN on MPP+ mediated neuroinflammatory response, SH-SY5Y cells were transfected with αSYN/STAT ODN and Scr ODN followed by MPP+ for 24 hours. The secretion of TNFα and IL6 were significantly inhibited in MPP+ stimulated SH-SY5Y cells by αSYN/STAT ODN transfection (Fig. 3). Scr ODN was similar to cells treated with MPP+.

DISCUSSION

The first peptide inhibitors of STAT proteins were discovered more than a decade ago, and attempts to target STAT signaling for therapeutic purposes are still ongoing [9]. Aberrant activation of the JAK/STAT pathway contributes to a number of autoimmune and neuroinflammatory diseases [10]. Several studies have illustrated that the novel inflammatory signals namely JAK/STAT, can be activated by LPS, TNF-α, IFN-γ, and IL-6 in the brain [11] and contribute to the pathogenesis of neuroinflammatory diseases [5]. The αSYN accumulation in the brain activated microglial and produced inflammatory cytokines or chemokines through the activation of the JAK/STAT pathway in different models of PD [12]. In addition, neurotoxin MPP+ treatment increased STAT1 expression levels and STAT1 phosphorylation and subsequent apoptosis in cerebellar granule neuron cells [13]. Furthermore, pyridone 6, a JAK inhibitor, reduced interferon β neurotoxicity in SH-SY5Y cells by reducing STAT1 and STAT3 phosphorylation and apoptosis [14].

Our research investigated the αSYN/STAT ODN protective effects on neurotoxicity in SH-SY5Y cells treated by MPP+. Our results exhibited that MPP+ exposure induced neuroinflammatory responses and apoptosis through the secretion of TNFα/IL6 and expression of cleaved PARP-1 in SH-SY5Y cells. In contrast, the transfected αSYN/STAT ODN reversed these changes caused by MPP+ in SH-SY5Y cells. These results strongly support the effectiveness of αSYN/STAT ODN, as the effect of Scr ODN was not observed.

Thus, gene therapy targeted to suppress mRNA level of SYN and transcription activity of STAT simultaneously might provide a new therapeutic strategy to prevent various neurological disorders.

ACKNOWLEDGMENTS

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (NRF-2021R1I1A1A01060030).

CONFLICTS OF INTEREST

Not applicable.

AUTHOR CONTRIBUTIONS

Investigation: Park J, Jang KM. Writing—original draft preparation: Park J, Jang KM. Writing—review: Jang KM. Funding acquisition: Park J. All authors have read and agreed to the published version of the manuscript.

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JIG Journal of Interdisciplinary Genomics

eISSN 2671-6771

Journal Information

Revised 3rd: June 23, 2023

Journal title, abbreviation, and acronym: Journal of Interdisciplinary Genomics (J Int Gen; JIG)
Publication start year: April 30, 2019
Publication frequency: Semiannual (April 30 and October 31)
Language: English
DOI prefix: 10.22742/jig
eISSN: 2671-6771 (Electronic only)
Electronic links: https://acoms.accesson.kr/jig
Country of publication: South Korea
Publisher: Interdisciplinary Society of Genetic & Genomic Medicine
Broad subject term(s): Genetics; Genomics; Medicine

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