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Novel HEXA Mutation and Its Impact on Hexosaminidase-A Activity in a Heterozygous Carrier: Insight from In-vitro and In-silico study

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Tay-Sachs disease (TSD) is a severe autosomal recessive neurodegenerative disorder resulting from mutations in the HEXA gene, leading to β-hexosaminidase A (Hex-A) deficiency. Accurate carrier identification through genetic screening is crucial for reproductive planning. This study aimed to characterize a novel HEXA variant found during preconception screening. A 30-year-old Iranian woman, whose husband is a known HEXA carrier, underwent whole-exome sequencing (WES). A novel heterozygous HEXA variant, c.[649T>C;650G>C] resulting in p.Trp203Pro, was identified in exon 6 and confirmed by Sanger sequencing. Its potential pathogenicity was assessed using in silico prediction tools (SIFT, PolyPhen-2, MutationTaster) and structural stability analysis (Molecular Dynamics and FoldX). Serum Hex-A enzyme activity was measured fluorometrically. The p.Trp203Pro variant was absent from ClinVar, HGMD, and gnomAD databases, indicating its novelty. In silico analyses consistently predicted the variant as "damaging" or "deleterious". Structural analysis predicted significant protein destabilization (ΔΔG +17.03 kcal/mol), comparable to many known pathogenic infantile-onset mutations. The patient serum Hex-A activity was 54.2% of total hexosaminidase activity, falling within the established range for heterozygous carriers. The comprehensive analysis strongly suggests that the novel p.Trp203Pro HEXA variant is likely pathogenic leading to reduced enzyme activity consistent with carrier status. This finding underscores the importance of thorough investigation of novel variants for accurate genetic counseling and highlights the expanding mutational spectrum of HEXA.

Introduction

Tay-Sachs disease (TSD) is a rare, autosomal recessive lysosomal storage disorder that leads to the progressive degeneration of the central nervous system (Fernández-Pereira et al., 2021). It arises from mutations in the HEXA gene, located on chromosome 15q23-q24, which encodes the alpha subunit of β-hexosaminidase A (Hex-A) (Dersh et al., 2016; Leal et al., 2020). Hex-A, a heterodimeric enzyme formed by alpha and beta subunits (the latter encoded by the HEXB gene), plays a critical role in lysosomal degradation of GM2 gangliosides, a class of glycosphingolipids abundant in neuronal membranes. When Hex-A

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activity is deficient due to recessive HEXA mutations, GM2 gangliosides accumulate in neurons, triggering severe neurological damage. This accumulation disrupts normal cellular function, leading to the hallmark symptoms of TSD, which vary in onset and severity depending on the nature of the genetic defect (Demir et al., 2020).

The clinical presentation of TSD is categorized into three primary forms. The infantile form, the most severe, typically manifests between 3 and 6 months of age (Bley et al., 2011). Affected infants exhibit early signs such as hypotonia, loss of developmental milestones, an exaggerated startle response, and a characteristic cherry-red spot in the macula, observable via ophthalmologic examination. This form progresses rapidly, with most children succumbing to the disease by 4–5 years of age due to respiratory failure or other complications (Bley et al., 2011). The juvenile form appears later, between 2 and 10 years, and is characterized by a slower progression (Specola et al., 1990). Symptoms include ataxia, dysarthria, spasticity, and cognitive decline, with patients typically surviving into adolescence (Specola et al., 1990). The late-onset form, known as LOTS, emerges in adolescence or adulthood and presents milder symptoms, such as muscle weakness, dystonia, and psychiatric disturbances, including psychosis or depression (Neudorfer et al., 2005). Individuals with LOTS may live into late adulthood, though the disease remains progressive. These variations in phenotype are largely attributed to the degree of residual Hex-A activity, with complete loss linked to infantile TSD and partial activity associated with milder forms.

The HEXA gene spans approximately 35 kilobases and comprises 14 exons (Wakamatsu et al., 1994). To date, over 100 mutations including point mutations, insertions, deletions, and splice site alterations have been documented. Most disease-causing mutations abolish Hex-A function entirely, resulting in the infantile phenotype, whereas certain missense mutations permit residual enzyme activity, correlating with juvenile or late-onset manifestations (Dersh et al., 2016). As an autosomal recessive disorder, TSD requires both HEXA alleles to be mutated for the disease to manifest. Heterozygous carriers, possessing one mutated allele, are asymptomatic but can transmit the mutation to their offspring (Gu et al., 2024). If two carriers reproduce, there is a 25% chance their child will inherit two mutated alleles and develop TSD.

The prevalence of TSD is notably higher in specific populations, such as those of Ashkenazi Jewish descent, where the carrier frequency is approximately 1 in 30, compared to 1 in 300 in the general population (Rozenberg & Pereira Lda, 2001). Similarly, while comprehensive epidemiological data for TSD across all regions of Iran may not be available, the recognized higher rates of consanguineous marriages in certain Iranian communities underscore the potential for an increased incidence of autosomal recessive disorders including Tay-Sachs disease (Jamali et al., 2014; Saadat et al., 2004). Given the devastating outcomes of TSD, preconception genetic screening is a vital tool for identifying carriers, particularly in these populations or among individuals with a family history of the disease (Rudnik-Schöneborn & Zerres, 2022). Screening typically combines enzyme assays, which measure Hex-A activity in serum or leukocytes, with genetic testing to pinpoint specific mutations. This approach empowers couples to make informed reproductive choices through genetic counseling, prenatal testing, or preimplantation genetic diagnosis, significantly reducing the incidence of TSD in screened populations (Richardson et al., 2022).

The present study focuses on the characterization of a novel heterozygous variant identified in the HEXA gene of a 30-year-old Iranian woman who underwent preconception genetic screening prompted by a family history of TSD on her husband's side, despite having no personal history of the disease. This variant, specifically two adjacent nucleotide substitutions, g.[72351197G>C;72351198T>C] on genomic reference NC_000015.10, corresponding to c.[649T>C;650G>C] (NM_000520.6) in exon 6 and resulting in a p.Trp203Pro missense change at the protein level, was initially detected by exome sequencing. The objective of this investigation is to describe this novel heterozygous mutation, confirm its presence via Sanger sequencing, analyze its potential pathogenicity using a combination of bioinformatics tools and protein structural analysis, and assess its impact on Hexosaminidase-A activity through in vitro enzyme assay. The findings aim to highlight the

implications of identifying such novel variants for genetic counseling, family planning, and the expanding understanding of the HEXA mutational spectrum.

Methods

Patient Background

A 30-year-old Iranian woman with no personal history of TSD, but with a family history of TSD in her husband's family, was referred for preconception genetic screening. Her husband is a known heterozygous carrier for a pathogenic HEXA variant. The screening was conducted to determine her carrier status for TSD as part of reproductive planning, given the autosomal recessive inheritance pattern of the disorder. Contextually, certain communities within Iran exhibit higher rates of consanguineous marriages, which can increase the prevalence of autosomal recessive conditions, underscoring the broader relevance of genetic screening in such populations.

Initial Genetic Screening

As part of a comprehensive preconception screening panel for recessive disorders, whole-exome sequencing (WES) was performed. Genomic DNA was extracted from peripheral blood leukocytes using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Library preparation and sequencing were conducted by Macrogen Inc. (Seoul, South Korea) on an Illumina NovaSeq 6000 platform, achieving a mean coverage depth of approximately 100×. Variant calling and annotation were subsequently performed using CLC Genomics Workbench version 24 (QIAGEN, Aarhus, Denmark). This analysis identified a heterozygous variant in the HEXA gene, described as NM_000520.6:c.[649T>C;650G>C], predicted to result in the protein change p.Trp203Pro.

Sanger Sequencing Confirmation

To confirm the heterozygous mutation identified by WES, Sanger sequencing was performed. Primers were designed to amplify exon 6 of the HEXA gene, where the variant is located. The forward primer sequence was 5'-ACATCGCAAGTTTGAGGC -3', and the reverse primer was 5'-AGACCCTGTTCTTGCCAG-3'. PCR amplification was carried out using Taq DNA polymerase, and the amplicons were sequenced bidirectionally by Macrogen Inc. Sequence data were analyzed using CLC Genomics Workbench version 24 to confirm the mutations.

Functional Bioinformatics Analysis

The potential pathogenicity of the p.Trp203Pro variant was evaluated using multiple in silico prediction tools: 1) The Sorting Intolerant From Tolerant (SIFT): Assesses the impact of amino acid substitutions on protein function based on sequence homology. 2) Polymorphism Phenotyping v2 (PolyPhen-2): Predicts the structural and functional effects of mutations using physical and evolutionary data. 3) MutationTaster: Evaluates pathogenicity by analyzing conservation, splice sites, and protein features. These tools were accessed via their web interfaces, and the variant was submitted for analysis.

Structural Analysis

The structural impact of the p.Trp203Pro mutation was assessed using the three-dimensional model of the native Hex-A protein obtained from the AlphaFold Protein Structure Database (UniProt ID: P06865) (Jumper et al., 2021). The p.Trp203Pro mutation was introduced into the WT structure using BIOVIA Discovery Studio (Dassault Systèmes, BIOVIA Discovery Studio, 2022). To preliminarily assess the immediate structural effects of the mutation and to obtain a refined starting structure, both the WT and mutant protein models underwent an energy minimization step. This was conducted via a 100 picosecond molecular dynamics (MD) simulation using the MD simulation engine within Molecular Operating Environment (MOE) 2024.06 (Chemical Computing Group ULC, Canada). Following this initial refinement, the stability of the mutant structure relative to the native protein was quantified using the FoldX5.1 plugin (Schymkowitz et al., 2005) as implemented in YASARA Model (version 25.1.13) (Krieger & Vriend, 2014). The difference in folding free energy (ΔΔG) between the mutant and WT structures was calculated using the FoldX stability evaluation function to predict the mutation's effect on protein folding and stability.

Measurement of Hexosaminidase-A Activity in Serum

Hexosaminidase A activity was measured in the patient serum using a fluorometric enzyme assay, a standard method for assessing HEXA function (Suzuki, 1987). Serum samples were incubated

with 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (4-MUG) at 37°C for 1 hour. The reaction was stopped with glycine-carbonate buffer (pH 10.0), and fluorescence was measured (excitation: 365 nm, emission: 450 nm). Hex-A activity was distinguished from total hexosaminidase activity by heat inactivation at 50°C for 3 hours, leveraging the heat stability of Hex B. Results were expressed as a percentage of total activity and compared to reference ranges for carriers and non-carriers.

Results

Confirmation of the Mutation

Sanger Sequencing

The heterozygous variant NM_000520.6:c.[649T>C;650G>C] in exon 6 of the HEXA gene, initially detected by whole-exome sequencing was subjected to confirmatory analysis by bidirectional Sanger sequencing. This targeted sequencing was performed on genomic DNA from the patient as detailed in Methods section 2.3. Examination of the resulting electropherograms provided unambiguous confirmation of the variant. Specifically, at nucleotide position c.649 (corresponding to genomic reference NC_000015.10, g.72351197 on the GRCh38 assembly), overlapping peaks for both thymine (T, representing the wild-type allele) and cytosine (C, representing the variant allele) were clearly visible (Fig. 1). Concurrently, at the adjacent nucleotide position c.650 (g.72351198), overlapping peaks for guanine (G, wild-type allele) and cytosine (C, variant allele) were also observed. These distinct double peak patterns at both nucleotide positions, consistently present in both the forward and reverse sequencing reads, are characteristic of a heterozygous genotype. Therefore, Sanger sequencing validated the initial WES finding and confirming the presence of the heterozygous c.[649T>C;650G>C] variant in the patient.

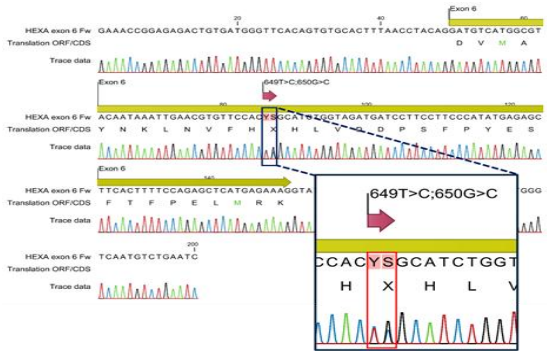


Fig.1. Sanger sequencing chromatograms of the HEXA gene spanning the exon 6 region. The forward sequence trace from the patient reveals heterozygous peaks: T/C (represented by IUB code Y) at nucleotide position c.649, and G/C (represented by IUB code S) at nucleotide position c.650. The amino acid translation of the corresponding exon 6 nucleotide sequence is presented below it. The inset shows a magnified view of the mutation site, highlighting the c.[649T>C;650G>C] variant.

Mutation Details

The confirmed variant consists of two adjacent nucleotide substitutions in exon 6 of the HEXA gene. According to the Human Genome Variation Society (HGVS) nomenclature guidelines (Rehm et al., 2013), this variant is designated as g.[72351197G>C;72351198T>C] based on the genomic reference sequence NC_000015.10. At the cDNA level, using transcript NM_000520.6, the variant is described as c.[649T>C;650G>C]. These tandem substitutions result in a missense change at the protein level, specifically p.Trp203Pro (Tryptophan to Proline at codon 203) in the alpha subunit of the β-hexosaminidase-A enzyme (UniProt ID: P06865).

To assess the novelty of this p.Trp203Pro variant, several publicly accessible human genetic variation databases were queried. The variant was not found in the ClinVar database (Landrum et al., 2018), the Human Gene Mutation Database (HGMD) Professional version 2024.1 (Stenson et al., 2020), nor was it reported in large population aggregate databases such as the Genome Aggregation Database (gnomAD v2.1.1 and v3.1.2) (Karczewski et al., 2020). The absence of this specific c.[649T>C;650G>C] (p.Trp203Pro) variant from these comprehensive databases strongly suggests that it is a novel mutation not previously documented in association with Tay-Sachs disease or in general population studies.

Bioinformatics Analysis

In-Silico Effect Prediction

To evaluate the potential functional impact of the novel p.Trp203Pro missense variant in the HEXA protein, several in silico prediction algorithms were employed as described in Methods section 2.4. The SIFT tool predicted the p.Trp203Pro substitution to be “damaging” with a score of 0.00 (scores < 0.05 are considered deleterious) (Ng & Henikoff, 2003). PolyPhen-2 also predicted this

variant as "probably damaging" with a high score of 1.000 (scores range from 0 to 1, with values closer to 1 indicating higher confidence in pathogenicity) (Adzhubei et al., 2010). Furthermore, MutationTaster 2025 analysis classified the p.Trp203Pro variant as "deleterious" with a Tree vote: 95|5 (del | benign), taking into account its effect on amino acid sequence, protein features, and evolutionary conservation (Steinhaus et al., 2021). Collectively, these in-silico predictions consistently suggest that the p.Trp203Pro substitution is likely to be detrimental to HEXA protein structure and/or function.

3.2.2. Protein Structure and Function Analysis

The p.Trp203Pro variant occurs within the alpha subunit of the β-hexosaminidase-A enzyme (UniProt ID: P06865). The tryptophan at position 203 (Trp203) is a relatively bulky, aromatic amino acid, while proline is known for its unique cyclic structure that can introduce conformational rigidity or kinks into polypeptide chains. To visualize and assess the structural consequences of this substitution, the p.Trp203Pro mutation was introduced into the AlphaFold-predicted native structure (Jumper et al., 2021) using BIOVIA Discovery Studio. Both WT and mutant models then underwent a 100 ps MD simulation and energy minimization using MOE, as detailed in Methods section 2.5. The resulting structures, with the location of the mutation highlighted, are shown in Fig. 2.

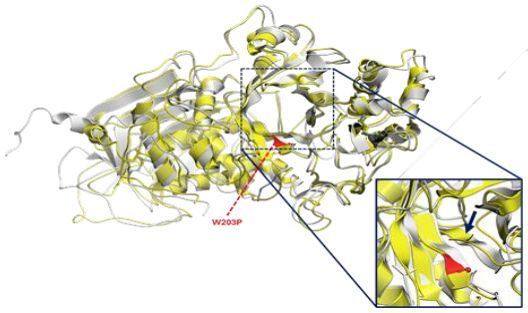


Fig. 2. Structural impact of the p.Trp203Pro mutation on the HEX-A protein. The image shows a superimposition of the WT HEX-A protein structure (light gray) and the p.Trp203Pro mutant structure (light yellow). Both structures represent the final frame of 100 picoseconds of energy minimization step of molecular dynamics simulation. The site of the p.Trp203Pro mutation is highlighted in red and indicated by a dashed line. The inset provides a magnified view of the region surrounding the mutation, illustrating local conformational changes and alterations to the secondary structure. These changes contribute to an overall structural deviation, as indicated by RMSD values (RMSD=0.774 Å). Notably, an arrow within the inset points to a truncated β-strand adjacent to the mutation site within the β-barrel motif of the enzyme.

The impact of the p.Trp203Pro substitution on protein stability was subsequently quantified by calculating the change in folding free energy (ΔΔG) using the FoldX5.1 plugin (Schymkowitz et al., 2005) within YASARA Model [17], based on the structures refined by MOE. The novel p.Trp203Pro variant yielded a predicted ΔΔG value of +17.03 kcal/mol. A positive ΔΔG value indicates that the mutation is predicted to destabilize the protein structure. This high ΔΔG value places the p.Trp203Pro variant among the more significantly destabilizing missense variants reported for HEXA. When contextualized with previously documented HEXA missense variants (a comparative analysis initially involved 51 such variants), a ΔΔG of +17.03 kcal/mol suggests a very substantial impact on protein stability. While some known severe pathogenic variants associated with infantile Tay-Sachs disease exhibit even higher positive ΔΔG values (e.g., p.Arg170Trp, ΔΔG = +29.4 kcal/mol; p.Ser210Phe, ΔΔG = +28.86 kcal/mol), the destabilization predicted for p.Trp203Pro (+17.03 kcal/mol) is considerably greater than many other mutations known to be pathogenic, including infantile forms such as p.Cys458Tyr (ΔΔG = +9.32 kcal/mol) and p.Glu482Lys (ΔΔG = +8.23 kcal/mol) (Table 1).

The significant destabilization indicated by a ΔΔG of +17.03 kcal/mol strongly suggests that the p.Trp203Pro substitution is highly likely to compromise the structural integrity of the HEXA alpha subunit. Such substantial structural perturbation (Fig. 2) could lead to various detrimental outcomes, including improper protein folding, increased susceptibility to proteolytic degradation within the cell, or altered conformational dynamics essential for correct substrate binding, catalytic activity, or the crucial heterodimerization with the beta subunit to form functional Hex-A enzyme. Consequently, these structural changes are anticipated to severely impair overall Hex-A enzyme function.

Hexosaminidase-A Activity Assay

To biochemically evaluate the functional consequence of the heterozygous c.[649T>C;650G>C] (p.Trp203Pro) *HEXA* variant, Hexosaminidase A (Hex-A) activity was measured in the patient's serum using a fluorometric enzyme assay with the 4-MUG substrate, and differentiated from total hexosaminidase activity by heat inactivation, as described in Methods section 2.6. The patient's serum Hex-A activity was found to be 54.2% of the total hexosaminidase activity. For context, the reference range for Hex-A activity in our laboratory is defined as >60% of total hexosaminidase activity for non-carriers, 40-60% for heterozygous carriers, and <15% for individuals affected with Tay-Sachs disease. The patient's Hex-A level of 54.2% falls unequivocally within the established range for heterozygous carriers of a *HEXA* mutation (Suzuki, 1987). This reduced enzyme activity, approximately 55% of the lower limit of the normal non-carrier range (if considering 60% as the lower normal limit, 54.2% is a reduction from the average normal which would be higher), is consistent with the presence of one functionally impaired *HEXA* allele due to the p.Trp203Pro variant, while the other allele presumably produces functional enzyme.

Table 1- Predicted change in folding free energy (ΔΔG) for the novel p.Trp203Pro variant compared to some selected known *HEXA* variants.

Variant ID	Amino Acid Change	Clinical Phenotype	ΔΔG (kcal/mol)
Novel Variant	p.Trp203Pro	(Under investigation)	+17.03
VAR_003207	p.Arg170Trp	Infantile	+29.4
VAR_003238	p.Cys458Tyr	Infantile	+9.32
VAR_003240	p.Glu482Lys	Infantile	+8.23
VAR_003246	p.Arg504His	Juvenile	+0.81
VAR_003210	p.Arg178Leu	Infantile	-0.14

Discussion

The precise characterization of novel genetic variants is crucial in medical genetics, especially for autosomal recessive conditions like Tay-Sachs disease, where carrier identification significantly influences reproductive decisions. This study focused on a novel heterozygous *HEXA* gene variant, c.[649T>C;650G>C] (p.Trp203Pro), found in an asymptomatic Iranian woman. Our analysis, encompassing genetic data, in-silico predictions, structural modeling, and biochemical assays, strongly supports the pathogenicity of this variant and confirms the patient's carrier status, providing essential information for genetic counseling.

The pathogenic nature of p.Trp203Pro was consistently indicated by computational tools (SIFT, PolyPhen-2, MutationTaster), all predicting a deleterious effect on protein function. Such agreement across different algorithms, which assess factors like amino acid conservation and physicochemical changes, bolsters the prediction's reliability (Ghosh et al., 2017). This was further corroborated by structural modeling, which predicted substantial destabilization of the Hex-A alpha subunit. The calculated change in folding free energy (ΔΔG) of +17.03 kcal/mol is highly indicative of a severe structural impact. Research utilizing protein stability predictors like FoldX, as used here, validates their effectiveness in distinguishing pathogenic missense variants, where ΔΔG values exceeding 1.5-2.5 kcal/mol are often strongly linked to pathogenicity (Gerasimavicius et al., 2020). The predicted destabilization for p.Trp203Pro is comparable to, or greater than, that of several known severe pathogenic *HEXA* mutations causing infantile TSD. Such severe destabilization likely compromises the alpha subunit's native conformation, potentially leading to misfolding, impaired dimerization with the beta subunit, or accelerated degradation via cellular quality control pathways like ERAD, ultimately diminishing the functional Hex-A enzyme pool (Dersh et al., 2016). Although Trp203 is not directly in the active site or GM2 activator protein binding loop, its location in the N-terminal domain means a proline-induced conformational change could globally affect protein integrity, as proline's rigidity often disrupts secondary structures.

Biochemical findings provided direct functional evidence. The patient's serum Hex-A activity was 54.2% of total hexosaminidase activity, a level characteristic of heterozygous TSD carriers who typically show 40-60% of normal enzyme activity due to one compromised allele (Mehta et al., 2016). This reduced activity confirms that the p.Trp203Pro variant, with a presumed normal allele, results in a phenotype consistent with TSD carrier status.

The clinical implications are significant. The patient sought preconception screening because her husband is a confirmed *HEXA* carrier. Confirming her carrier status for p.Trp203Pro

establishes a 25% risk in each pregnancy of having a child with TSD. This information is vital for informed reproductive choices, such as prenatal diagnosis or PGD (Edwards & Laing, 2022).

This novel pathogenic variant discovery in an Iranian individual contributes to understanding the *HEXA* mutation spectrum in this population. Previous studies, including those by Jamali et al. (Jamali et al., 2014) and Haghighi et al. (Haghighi et al., 2011), have highlighted genetic heterogeneity for TSD in Iran. The higher rates of consanguineous marriages in some Iranian communities, known to increase autosomal recessive disorder incidence, further emphasize the need for comprehensive, population-relevant carrier screening (Saadat et al., 2004). Discovering new variants like p.Trp203Pro underscores the necessity of sequencing-based screening that detects both known and novel mutations, rather than relying solely on panels developed for other populations.

Our study's strength is its comprehensive methodology, aligning with ACMG variant interpretation guidelines (Richards et al., 2015). However, limitations include the assessment being based on a single carrier and primarily in-silico or in-vitro data. Definitive confirmation would benefit from functional studies expressing p.Trp203Pro in a cellular model to assess protein folding, trafficking, stability, and catalytic activity. Family segregation analysis, if possible, could also offer support. Future research should prioritize such functional characterization. Screening for p.Trp203Pro in larger Iranian cohorts, especially those undergoing preconception screening or with suspected TSD, would help determine its prevalence and inform regional genetic screening panel design.

Conclusion

This investigation successfully identified and characterized a novel heterozygous *HEXA* gene variant, c.[649T>C;650G>C] (p.Trp203Pro), in an asymptomatic individual through preconception screening. Comprehensive in silico analysis predicted this variant to be damaging, and structural modeling indicated significant protein destabilization, ranking it among the more severely impacting known *HEXA* missense mutations. Crucially, biochemical assays confirmed a reduction in Hexosaminidase-A enzyme activity to levels consistent with a Tay-Sachs disease carrier state. This multi-faceted approach underscores the variant's likely pathogenicity and highlights the critical role of such comprehensive evaluations for accurate genetic counseling and informed reproductive decision-making, particularly when novel genetic findings emerge.

Ethical Statement

This study was conducted in accordance with the principles of the Declaration of Helsinki. The patient provided written informed consent for the genetic analysis and for her anonymized data to be used in this research and subsequent publication. All procedures were part of the patient's standard clinical genetic screening process, and efforts were made to ensure confidentiality.

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