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MOLECULAR GENETIC STUDY OF AUTISM SPECTRUM DISORDER IN KHYBER PAKHTUNKHWA, PAKISTAN

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ABSTRACT

Autism spectrum disorder (ASD) is a complex neurodevelopmental disorder with a strong genetic component in which rare variants contribute significantly to risk. Around 1-3% of world population are affected, and the ratio were higher in developing as compared to developed countries due to unawareness, health problems, poverty, and socio-economic problems. Out of six families, only one family was shortlisted and was recruited from Nowshera, Khyber Pakhtunkhwa province of Pakistan. Whole Exome Sequencing (WES) was performed and

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identified variant in shortlisted ASD family. WES Identify the possible causative gene RAS Activating protein Like 2 *(RASAL2)* gene. Furthermore, Sanger sequencing was performed for the confirmation of variant identified in the *RASAL2* gene. After genetic analysis, clinical features were compared and to be found relative as studied in the literature. Pathogenicity of this mutation was evaluated using algorithmic mutation prediction tool. WES revealed a reported missense mutation in *RASAL2* gen present on chromosome 1 in the exon 5 at c.638G>C which ultimately altered the amino acid serine to threonine at position 213. Moreover, mutation in the genes lead to disrupt the protein structure which involves in regulation of upstream and downstream pathway. In conclusion, WES was used to identify the genetic alteration in family with ASD. Mutation in *RASAL2* were uncovered and clinical features of cross checking uncovered that family had ASD.

Introduction

Autism spectrum disorder (ASD) is a complex neurodevelopmental condition with a strong genetic basis, where rare variants significantly contribute to disease risk (Lord et al., 2020; Parenti et al., 2020). The global prevalence of ASD affects approximately 1-3% of the population (Salari et al., 2022), with higher reported rates in developing countries compared to developed nations. This disparity is attributed to factors including limited awareness, healthcare access challenges, poverty, and socioeconomic barriers (Bölte et al., 2019; Volk & Sheridan, 2020).

In this study, we investigated a consanguineous Pakistani family with ASD from Nowshera, Khyber Pakhtunkhwa province. Consanguinity represents a significant risk factor for autosomal recessive neurodevelopmental disorders in Pakistani populations (Ilyas et al., 2020; Bittles & Black, 2010). From an initial screening of six families, one multiplex family was selected for comprehensive genetic analysis. Whole exome sequencing (WES) was employed to identify potential causative variants (Savatt & Myers, 2021), followed by Sanger sequencing for validation (Grimberg et al., 1989).

WES analysis revealed a missense mutation in the RAS Activating protein Like 2 (*RASAL2*) gene, located on chromosome 1. The variant (c.638G>C; p.Ser213Thr)

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resides in exon 5 and results in a serine-to-threonine substitution at amino acid position 213. This alteration is predicted to disrupt protein structure and function. *RASAL2* encodes a GTPase-activating protein (GAP) that plays a critical role in regulating RAS signaling pathways (Donovan et al., 2002), which are essential for neuronal development, synaptic plasticity, and cellular proliferation—processes frequently implicated in ASD pathogenesis (Guo et al., 2020; Reichova et al., 2018). Dysregulation of Rho GTPase signaling has been increasingly recognized in motor disorders and neurodevelopmental pathologies (Bloch-Gallego & Anderson, 2023).

Computational pathogenicity prediction tools supported the deleterious nature of this variant. The identified phenotype-genotype correlation was consistent with previously reported ASD clinical features in the literature (Faras et al., 2010). The RAS signaling pathway represents an important biological mechanism underlying neurodevelopmental disorders, and disruption of its tight regulation through *RASAL2* mutations may contribute to ASD susceptibility (Donovan et al., 2002; Simanshu et al., 2017).

This study demonstrates the utility of WES in identifying rare genetic variants in familial ASD cases (Savatt & Myers, 2021; Najmabadi et al., 2011). The discovery of a pathogenic *RASAL2* mutation in this Pakistani family expands the genetic spectrum of ASD and highlights the importance of investigating diverse populations to uncover novel disease-associated variants (Ilyas et al., 2020; Yaqoob et al., 2004). These findings contribute to our understanding of ASD etiology and may inform future diagnostic and therapeutic strategies targeting the RAS signaling pathway (Simanshu et al., 2017; Salcedo-Arellano et al., 2021).

Methodology

Identification of Families

Six families were identified in MARDAN and NOWSHEHRA (PAKISTAN) were the subjects of this investigation. Families with two or more probands were found by researchers. While some probands displayed non-syndromic characteristics, others had syndromic characteristics, such as abnormal behavior and low cognitive development linked to the symptoms and signs of ID and ASD. The researchers went to residence of affected families to collect samples and to

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obtained relevant information regarding family, onset of disease, the number of family members affected, the severity of disease, and other abnormalities related to the disease.

Ethical Approval and Consent Form

The parents of probands signed a written form prior to the collection of blood samples. The purpose of the research was clearly described. Informed consents were obtained from elder members of the families participating in this research study. This research study was approved by ethical review committee of International Islamic University Islamabad, Pakistan.

Clinical Features

Clinical features that help us distinguish between syndromic and non-syndromic ID and ASD were reported from the probands. These included things like sex, age, height, head postures, strabismus, facial dysmorphism, consanguinity, developmental delay, birth, pregnancy period, sleeplessness, ethnicity, talking walking and schooling.

Drawing Pedigree and Analysis

Researcher drawn the pedigree after collecting the information of identified families. By drawing pedigrees, the males were represented by a square and females by circular symbols. For the affected males and females, the squares and circles were shaded completely. While normal individuals were shown as unfilled symbols. Deceased individuals were represented by drawing a diagonal line across a square/circle to distinguish them from alive individuals. Consanguinity was represented by a double line between couples. Each generation was represented by Roman numerals (I, II), while number of individuals by Arabic numerals (1, 2). All the pedigrees were drawn through "Haplopainter" software after collecting data about the thorough medical history of the families. On the basis of material obtained by questioning the elder members of the families, pedigrees were constructed. The purpose of constructing pedigrees was to understand the mode of inheritance of the disease and genetic relationships among the family members.

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Blood Collection

Peripheral blood was drawn from the affected individuals along with their parents and siblings, with the help of 5 ml-disposable syringes and the blood was transferred into standard potassium EDTA tubes to prevent clotting. The collected samples were stored at 4 °C in Bio-Medical Engineering Lab (BMEL) at the Department of Biological Sciences, International Islamic University Islamabad, Pakistan.

Lab Work

Lab work includes DNA extraction from blood and quantification, electronic pedigree drawing, Whole Exome Sequencing data analysis.

DNA Extraction (Inorganic Method)

Genomic DNA was isolated from the blood samples following non-organic method (Grimberg et al., 1989).

Required Materials

Proteinase-K, SDS, chilled isopropanol, ethanol, Tris-EDTA buffer, Falcon Tubes, Eppendorf tubes, pipette, TNE, Incubator, saturated NaCl (6M), Freezer, Centrifuge Machine & the compositions of these solutions are shown in Table: 01

Procedure

1000µl blood from each sample was taken in separated 15ml falcon tubes. Blood samples were thawed for the RBCs lysis. Washing/T.E/Lysis buffer (10mM Tris HCl pH8, 2mM EDTA) was added to each sample by raising the volume up to 15 ml in falcon tubes. These were centrifuged at 3000 rpm in the Beckman TJ-6 Centrifuge for 20 minutes at 25°C. Supernatant was discarded using pipette and pellet was broken by gentle tapping and vertexing. Washing was repeated for three to four times till the WBCs' pellet got free of hemoglobin (RBCs). Supernatant was discarded up till 2ml mark of falcon tube. The pellets were resuspended in proteins' digestion mixture. Each tube is mixed with 1ml of TNE buffer (1OmM Tris HCl pH8, 2mM EDTA, 400mM NaCI), 10µl of Proteinase K (10 ug/ul) and 30µl of 10% SDS (each for 1000µl of blood volume). The samples were mixed gently for 30 seconds and incubated at 50°C in incubator for 1 hour.

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After that samples were taken out and 200µl of saturated NaCl (6M) was added in each tube. Sample tubes were shaken vigorously by hands and then placed in freezer for 20 -30 minutes. The samples were taken out from freezer and left at room temperature for melting. The sample tubes were centrifuged at 3000 rpm for 30 min at 25°C. 1.5ml supernatant from each sample was taken out in a separate tube and the protein pellet was discarded. DNA was precipitated in the form of white threads from the supernatant by gently inverting the tubes after adding equal volume of isopropanol. After 10 minutes samples were centrifuged at 3000 rpm for 15 minutes at 25 °C. Supernatant was discarded carefully and pellet was dried at room temperature for 1.5 hrs. Afterwards, 200µl of absolute ethanol was added in each tube, and centrifuge at 3000 rpm for 15min to remove any remaining protein or other unwanted components. Finally, the supernatant was discarded and pellet containing DNA, left in each tube.

DNA was then dissolved in 100µl (for each 1000µl volume of blood) of autoclaved TE Buffer/Elution Buffer (Tris 10 mM, EDTA 0.1 mM). The samples were kept in an incubator at a temperature of 70°C to inactivate any remaining nucleases. All the samples were transferred into properly labeled Eppendorf tubes and stored in freezer at 4 °C.

Table 1: ***Chemical Composition for solutions used in DNA extraction***

Solutions	Chemical Compositions
Tris EDTA (TE) Buffer	Prepare 800 mL of distilled water in a suitable container. Add 15.759 g of Tris-HCl (desired pH) to the solution. Add 2.92 g of EDTA (pH 8) to the solution.
TNE	5ml Tris HCl mixed in 470ml distal water. Add 3ml plus 340 µl NaCl. Then add in distal water. Add 20 µl EDTA
10% SDS	10g in 50ml water
Proteinase K	2.5ml EDTA & 2.5 ml Glycerol for 100mg P.K

Quantification of DNA Samples

DNA samples were quantified by agarose gel electrophoresis. DNA quality was checked through 1% agarose gel electrophoresis.

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Preparation of Agarose Gel

A conical flask was taken. 60 ml 1×TBE buffer and 0.6g agarose powder was added to it. The solution was made by shaking the flask thoroughly. The flask was heated on micro-oven for 30 seconds to 2 minute until bubbles appeared. The solution was cooled for 1 to 2 minutes. 2 ul Ethidium Bromide was added to it. The solution was then poured into a clean gel-caster containing a comb. After 30-35 minutes the gel was prepared in semi-solid form. The gel containing wells was ready for electrophoresis process.

Agarose Gel Electrophoresis

This method uses the UV- induced fluorescence of Ethidium Bromide dye intercalated into the nucleic acid. The amount of fluorescence is proportional to the amount of nucleic acid present. Fluorescence of the test DNA and that of a standard DNA was compared. % Agarose gel slab (stained with 2ul Ethidium Bromide) was prepared. Gel was loaded by 5ul of each DNA sample mixed with 2ul of loading dye (Bromophenol blue 2X) with the help of micro-pipette. Loaded mixture was run on 120 V for 35 minutes. The loaded samples containing DNA were moved from negative end to the positive end of the gel-tank, because of the negative charge on DNA molecule. When the samples reached to the positive pole of the gel-tank, the gel was removed. The gel slab was observed in gel documentation apparatus (UV trans- illuminator) by using Clinx GenoSens software. The samples' quantity was estimated and recorded.

Table 2: ***Composition of solutions used in Gel Electrophoresis***

Solutions	**Compositions**
Gel Loading Dye	
TBE Buffer	Tris 0.89 M
	Boric Acid 0.025 M
	0.5 M (pH 8.3) EDTA

Visualization of DNA

The gel was carefully placed into a Gel-Doc tool containing ultraviolet light. Photograph were capture in a computer linked to the tool. Sharpen bands were appeared. Sharpen bands meant high quality DNA was extracted.

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Performing NanoDrop

DNA concentration and purity were determined using the NanoDrop (SenseTech) spectrophotometer from ThermoFisher Scientific. The process typically involved preparing a small volume of the DNA sample and pipetting it onto a clean pedestal on the instrument. Afterwards appropriate setting was adjusted for nucleic acids and blanked the instrument using a reference solution. After closing the measurement arm, then the NanoDrop measured the absorbance of ultraviolet (UV) light by the sample and calculated the DNA concentration based on established algorithms. The instrument provided results in terms of concentration (e.g., ng/µL) and could offer information on sample purity through metrics such as the A260/A280 ratio, which assessed the presence of contaminants like proteins. One shortlisted ASD family's DNA (proband, father, mother) were sent to Canada for Whole Exome Sequencing.

Whole Exome Sequencing

Whole Exome Sequencing method provides comprehensive analysis of an individual's exome, the region of the genome that codes for proteins. Despite the genome's vast amount of information, most data comprised of noncoding regions that are not directly involved in protein synthesis. Exomes represent only about 1-2% of the genome, but they contain most of the disease-related variations.

Sequencing and Data Generation

NextSeq550 series sequencer was used for sequencing of prepared exomes libraries. Sequencer generated vast amounts of raw data in the form of short reads. These short reads represent the nucleotide sequences of the exonic regions under investigation. The high-throughput nature of Illumina sequencing allows for the generation of millions of these reads, providing a comprehensive dataset for subsequent analysis. Finally, all reads data are generated in the FASTQ file which was further analyzed for the identification of variants.

Bioinformatics Analysis

Processing of FastQ file, annotation and variant calling, and variant prioritization are the major steps in NGS data analysis.

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Processing of FastQ file

The data generated by Illumina sequencer is in the form FastQ file. FASTQ format is a text- based format for storing both a biological sequence (usually nucleotide sequence) and its corresponding quality scores. Furthermore, the FastQ file is processed through software called Sentieon. The Sentieon DNAseq software is a package of tools used to perform ultrafast variant detection in genomic data obtained from NGS.

Sentieon is capable of processing FASTQ files, although it does not directly convert them into BAM and VCF formats. Instead, it serves as a comprehensive suite for next-generation sequencing (NGS) data analysis, encompassing various steps that involve the use of external tools such as BWA and GATK. The workflow begins with alignment, where Sentieon employs BWA-MEM to map short sequences (reads) from FASTQ files onto a reference genome. This established the precise genomic locations to which each read aligns, forming the basis for subsequent variant calling. Following alignment, Sentieon identifies and marks duplicate reads resulting from PCR amplification, thereby reducing bias and enhancing the accuracy of variant calling. The process continues with indels realignment, utilizing tools like GATK's IndelRealigner to refine alignments around small insertions and deletions (indels), thereby improving accuracy in variant calling within these regions. Sentieon also performs base quality score recalibration (BQSR) to adjust base quality scores in FASTQ files, ensuring accurate quality information for variant calling. Subsequently, the raw variant calls undergo VCF formatting and filtering through GATK's Variant Filtration and VQSR tools, respectively. This step eliminates unreliable variants and enhances the overall quality of the resulting VCF file.

Annotation and Variant Calling

Annotation refers to the process of assigning biological information to genomic features such as genes, transcripts, and variants. The annotation process involves comparing the genomic features to a reference database to identify their biological function, location, and other relevant information. While Variant calling is the process of identifying genetic variations such as single nucleotide

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polymorphisms (SNPs) and small insertions and deletions (indels) from next-generation sequencing data. Both annotation and variant calling were processed using a software named VarSeq that streamlines the process of annotating and filtering variants obtained from next-generation sequencing pipelines. The software accepts a variety of input formats, including VCF, BED, and GFF3.

The annotation process in VarSeq is a multi-step process that involves importing variant data, normalizing the data, and annotating the variants with relevant information. The database provides a variety of annotation tracks, including allele frequency, functional prediction, and gene annotations. Every gene track available in VarSeq includes a transcript annotation algorithm that closely matches Ensembl's Variant Effect Predictor's behavior, with some additions to aid the clinical interpretation process.

In VarSeq, variant calling is performed using the Genome Analysis Tool-Kit (GATK) best practices pipeline for germinal variant calling. The software also provides a variety of filtering options, including filtering by gene, variant type, and functional impact. Finally, the annotated file are exported in the Excel spreadsheet for further variant prioritization.

Variant Prioritization

In NGS Data analysis, variant prioritization plays a crucial role of sifting, a vast number of identified genetic variants (mutations) and ranking them based on their likelihood of being disease-causing or relevant to the research question. Trios (probands, mother, and father) information were merge in the excel file and then open in the Excel spreadsheet where various filters were applied via existing pedigree information either Recessive, Dominant, X-linked and De-novo. Various sections of trios are present containing filters through which variants are filtered out. Various sections includes; variant information, probands, mother, father, match ACMG, RefSeqGenes, gnomAD Exome Variants, ClinVar, Missense Badness. Variant information contains chromosome positions, and also mention the reference and altered nucleotide which exhibits i.e: C/T.

Firstly, zygosity was check out via present pedigree which were drawn after taking the family information whether it is homozygous or heterozygous. In this

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study, the exist pedigree exhibit that parents were homozygous while the probands is heterozygous. Once the filter is applied, the variants were more filtered out. Secondly, in the RefSeqGenes section, sequence was filtered out by selecting the frameshift variant, inframe deletion, inframe insertion, and missense variant. After filtration, the variants are further shortlisted as strong candidates for causing disease. Thirdly, compare the gene with the gnomAD for their allelic frequency. Higher allelic frequencies are excluded and considered as polymorphism while lower allelic frequencies represents the possible genes for causing disease. Fourthly, ClinVar section, is an NCBI tool, showed the pathogenicity of shortlisted genes. Fifthly, missense Badness section was automatically generated the score, that how much the genes is possible to cause the disease. Finally, the most causing disease gene was selected and further studied their functions using various databases like OMIM, Genecard, and Varsome.

Validation by Sanger Sequencing

In the current study, specific shortlisted gene are further validated by Sanger sequencing method. Sanger sequencing is a DNA sequencing technique that determines a DNA molecule's nucleotide order by using chain termination. This process involves denaturing the DNA, annealing a primer, and synthesizing DNA strands with a mixture of regular deoxynucleotides (dNTPs) and chain-terminating dideoxynucleotides (ddNTPs). By using capillary electrophoresis, separate the terminated fragments based on size, the sequence can be identified by examining the fluorescently labeled chromatogram that is produced.

Primer Designing

Primer was designed using Primer3Plus software (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Both primers, forward and reverse, length was 20 base pairs, Tm was 59.8°C and 58.7°C, GC-Content was 50% and 50% respectively. Both primers had low self-complementarity values and had negative results for primer dimerization. Cross checking of both primers was carried out by a software named BLAT (Basic Local Alignment Tool) and UCSC (University of California and Santa Cruz) (https://genome.ucsc.edu/cgi-bin/hgBlat, https://genome.ucsc.edu/cgi-

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bin/hgPcr).

Polymerase Chain Reaction (PCR) based Amplification

PCR was performed for conformation of particular gene identified in NGS data analysis. PCR reagents are given in the Table (3). A thermal cycler (AMPLICON PTC-15) has been used to amplify polymerase chain reaction (PCR). The reaction mixture was thoroughly mixed and incorporated into PCR sources for amplification (Table 3). PCR was scheduled for 35 cycles. System conditions included 94 °C for 5 minutes to produce a DNA model followed by 35 cycles. Each cycle had three steps: 94 °C for 45 seconds of denaturation fluctuation, 55 °C for 45 seconds for drag, and 72 °C for expansion. Then 72 °C for 10 minutes was set for the final expansion and finally, 4 °C for 4 minutes was needed to cool the machine. PCR products were checked for amplification on 2% agarose gel.

After purification, PCR amplicon undergo Sanger sequencing. Volume of 25 µl using FastStartTM Taq DNA Polymerase, 5 U/µl Kit (Roche), 0.5 µl of each primer with a concentration of 10 pmol/µl (Eurofins Scientific, Luxembourg), 1 µl of dNTPs (2.5 mM), and 2 µl genomic DNA in a concentration of approximately 50 ng/µl were used to perform PCR. A mixture of Exonuclease I 20 U/µl and FastAP Thermosensitive Alkaline Phosphatase 1 U/µl (Thermo Fisher Scientific, United States) was used to purify the amplicons. Purified PCR products were sequenced using the BigDye Terminator Kit v1.1 (Thermo Fisher Scientific, United States) and ABI 3500Dx Sequencer (Applied Biosystems). Fluorescently labeled ddNTPs were detected when pass through capillary electrophoresis and chromatogram was obtained.

Table 3: ***PCR Reaction Mixture***

Components	**20 µl Reaction Mixture**
One Taq 2x Master Mix with Standard Buffer	12µl
Forward Primer	1µl
Reverse Primer	1µl

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Template DNA	2µl
Nuclease-Free Water	4µl

Table 4: ***PCR outline for DNA amplification***

Steps	Temp°C	Duration	No. of cycles
Denaturation	94	5min	1x
Denaturation	94	45sec	
Annealing	55	45sec	35x
Elongation	72	45sec	
Final elongation	72	10min	1x

Co-segregation study

The study was performed to evaluate the co-relation between the gene locus and NDD disorder in the family as depicted in the pedigree. The analysis was performed to track the inheritance variant within a family pedigree, examining how often the variant appear alongside the trait in different generation. It provide strong evidence of variant involved in causing the disease, if variant co-segregate with the disease across generation.

Results

During field work, out of six families, only one ASD family was shortlisted for genetic mapping. The particular family having at least one individual affected with Autism spectrum disorder (ASD). The family showed symptoms of ASD and were diagnosed by a Pediatrician and psychiatrists. This family were included in the study with prior consent of all the family members. Blood samples were taken from affected individuals, their parents and also from neurotypical sibling in few cases for isolation of DNA which was used for their genetic analysis.

ASD Pedigree Explanation

This family was enrolled from district Nowshera, Khyber Pakhtunkhwa, Pakistan. It is a Pathan origin family with consanguineous marriages in last two generations with no family history of ASD. The family has two sons, the affected child is an older 7-year-old boy and the other is 4 years old neurotypical. The affected individual was diagnosed with ASD at the age of 3.10 years by a certified

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podiatrist in Pakistan. Blood was collected from both parents and Affected individual for WES screening.

Clinical Record

Clinical manifestations include delayed communication, behavioral and social challenges, fleeting eye contact, poor responses to his name and reciprocal communication, Repetitive Behaviors, need-based social communication, struggles with play and social skills, lack of concentration, sensory problems, lacking some basic self-care abilities, such as not being properly trained for the toilets, enhanced screen use, and hyperactivity. He is good in mobility. He was good with intelligence and memory. No seizers, no depression, no hearing impairments, and no gastrointestinal issues were noticed. He is verbal and can speck but slow. Presently he is showing a slow improving with speaking skills. From history provided, assessment and ADOS 2 he displayed very significant impairments with his social communications/interactions along with repetitive behaviors and sensory issues and these abnormalities meet the criteria for Autism Spectrum Disorder on DSM 5. He also displayed associated significant developmental delay in most areas. Patient was diagnosed ASD at the age of 46 months in a couple of certified ASD assessment clinic.

Regression in speech and social reciprocity were observed by parents at the age 2.5 years. The developmental stage noticed are: Neck holding from 3rd month, Early sat and walked at 12th month. Social smile 3-12 months, vocalized 5-12 and babbled and using single words at 12th month. No concerns in first 2 years. Born term by section because of premature rupture of membranes (PROM) after normal pregnancy. No issues at Birth, and No illness.

Molecular Genetic Studies

Genetic Finding

WES was performed for all trios of PKASD. A novel heterozygous missense mutation is identified at Chr: 1(1:178389719), NM_170692.4:c.638G>C, NP_733793.2:p.Ser213Thr in the exon five (5) of RAS protein activator like-2, ***(RASAL2)*** gene. Co-segregation analysis was performed and the mutation segregated successfully. The mutation was heterozygous in the affected

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individual and homozygous in the parents. The variant have very low frequency (0.000007987) in gnomeAD which predicts that it is a rare mutation and the Pakistani population is unexplored so far.

In-silico Analysis

To find out whether the mutation is pathogenic or not, in-silico analysis was undertaken using various mutation prediction software such as SIFT, Polyphen-2, Mutation Taster, PROVEAN, MuPro, and I-Mutant. These mutation prediction tools predicted that GTPase activating protein mutation is deleterious, probably damaging, disease causing, Neutral, and DECREASE stability.

Table 5: ***Illustrate all the results of in-silico analysis performed for this study***

Family	**PK-ASD**
Nucleotide variant	c.638G>C
Protein variant	p.S213T
Status	Heterozygous
Type of mutation	Missense
Previously reported	Novel
Polyphen-2	PD(0.974)
Mutation Taster	DC(0.999)
SIFT	Dam(0.03)
PROVEAN	Neutral
MuPro	DS(-1.08)
I-Mutant2.0	DS(-1.08)

PD=probably damaging, DC=disease causing, Dam=damaging, DS=decrease stability

Sanger's Result

Sanger sequencing was performed to confirm the variant identified through NGS in the trio (proband, father, and mother). The variation (G/C alteration) also seen in chromatogram obtained from Sanger sequencing. The chromatogram's result for father and mother showed that both are homozygous (GG) and (GG) respectively, at 638 position according NM_170692.4:c. While

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the chromatogram of affected individual had heterozygous (GC) for that particular position as shown in figure 1.

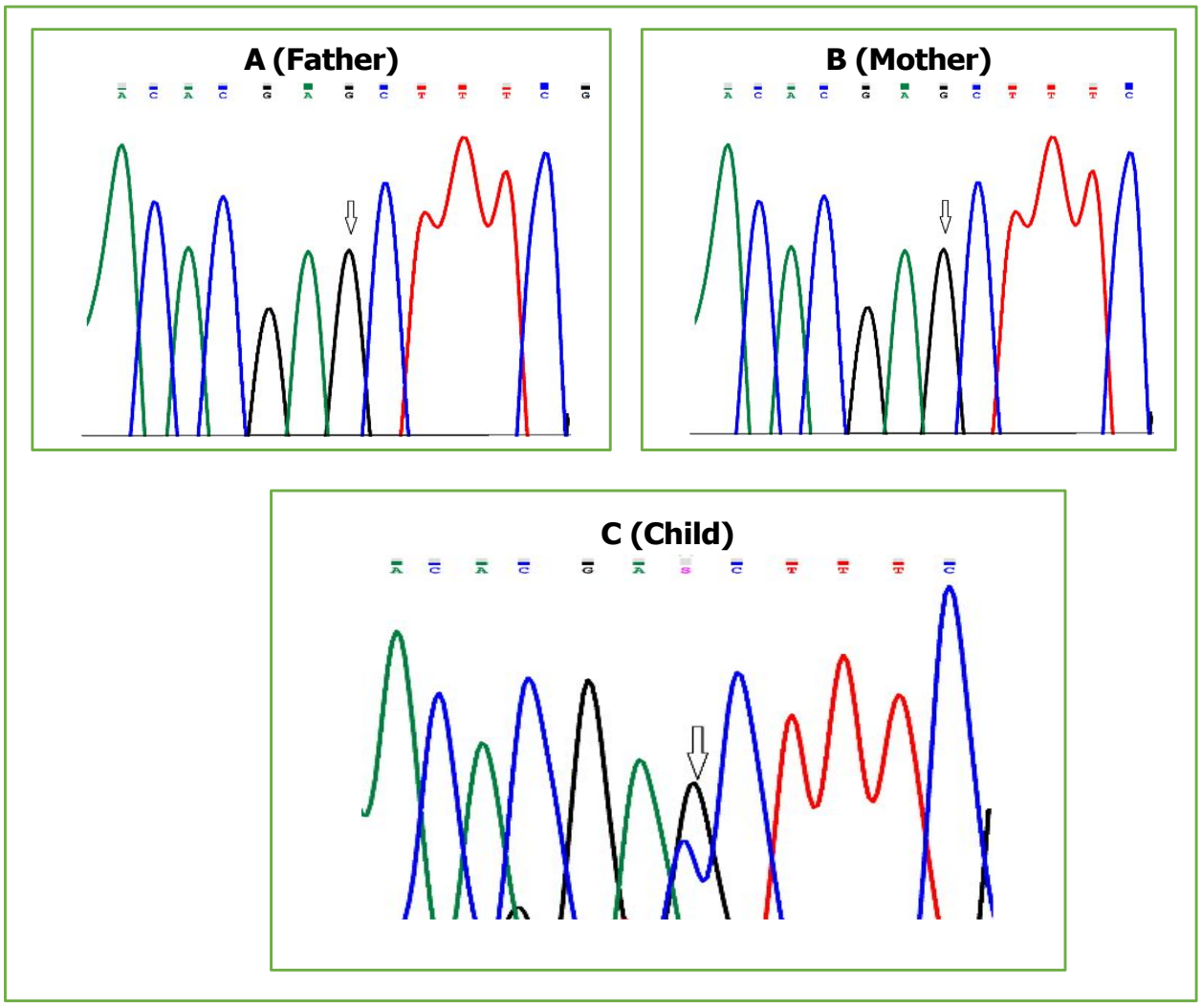


Figure 1 Chromatogram of trios ((A) Father, (B) Mother, (C) Child)

Co-segregation Analysis

In this study, co-segregation analysis played a pivotal role in predicting the genetic basis of the observed traits within affected individuals. Through a comphrensive examination of familial inheritance patterns, we successfully identified a specific genetic variant that co-segregated with trait of interest. We were able to examine this variant's inheritance in affected individual over several generations by using co-segregation analysis, and the results strongly suggest that this variant is related to the phenotype reported. The variant substitution

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(G/C) found in exon 5 of the RASAL2 gene recognized as a significant candidate in the etiology of the trait. This finding highlights the possible functional significance of this genomic area in addition to highlighting a strong correlation between the genetic mutation and the phenotypes. Co-segregation and analysis of the family studied in this research

Conserved Region

Conserved regions across different species provide a significant evidence that play a vital in maintaining a specific function. To better comprehend evolutionary links between different species, conserved regions are considered. After NGS analysis, mutation was reported at chromosome 1 in the exon 5, coding region at c.638G>C, is predicted to be a missense mutation which ultimately changed the protein sequence. Due to missense mutation at coding region (c.638G>C) that can change the amino acid serine to threonine in the protein sequence position 213 in the exon 5(coding region). Multiple sequence alignment of *RASAL2* protein was performed by using ClustalW tool, orthologs showed that serine were highly conserved among species as shown in figure 2.

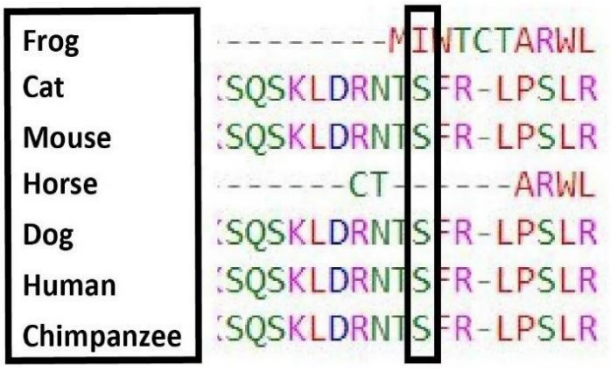


Figure 2 Conserved region among frog, cat, mouse, Horse, dog, human, chimpanzee shows the importance of serine amino acid

Protein Structural Analysis

GAP protein structure was predicated by using different bioinformatics tools and software's. Sequences of 1280 amino acid were retrieved from NCBI (https://www.ncbi.nlm.nih.gov/nuccore/NM_170692.4) in FASTA format. For three dimensional structure prediction for both normal and mutated GAP

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protein Comparative modeling was employed by using SWISS MODEL. Serine ($C_3H_7NO_3$) belongs to polar R group which is not essential in mammals replace with Threonine ($C_4H_9NO_3$) belongs to polar, charged R group which is essential in mammals. Both amino acids differ in isoelectric point (5.68 and 5.60) and mass molar (105.09 g/mol and 119.12 g/mol). Membrane topology of human GTPase activating protein was established and visualized with PyMOL. Sheet, loop, and helix protein was observed in different colour.

Discussion

The term "neurodevelopmental disabilities" (NDD) describes long-term conditions that affect central nervous system (CNS) functions such as motor skills, cognitive capacities, behavior, and/or communication in early childhood (Lord et al., 2020; Parenti et al., 2020). Intellectual disability (ID) and Autism Spectrum Disorder (ASD) are two major diseases in this domain that are characterized by lower ability to acquire new experience and facing difficulty in learning and social interaction, respectively (Faras et al., 2010; Lord et al., 2020; Ropers, 2008). According to Bölte et al. (2019), approximately 1% to 3% of the world's population suffer from both ID and ASD due to genetic and environmental conditions. Similarly, Zeidan et al. (2022) and Chiarotti and Venerosi (2020) reported comparable prevalence rates in their systematic reviews. The ratio of genetic factors as compared to environmental contribution is high in both ID and ASD (Bölte et al., 2019; Volk & Sheridan, 2020; Parenti et al., 2020).

Polygenic nature showed that large number of genes are responsible in both ID and ASD (Parenti et al., 2020; Ropers, 2008; Ramaswami & Geschwind, 2018). According to Najmabadi et al. (2011), approximately 700 genes are involved in causing ID and around 800 genes contribute in development of ASD. Several families were identified with both ID and ASD in Pakistani population (Suhaib et al., 2019; Durkin et al., 1998; Ilyas et al., 2020; Kanwal et al., 2015; Yaqoob et al., 2004). Zhang et al. (2019) investigated Next Generation Sequencing approaches using DNA isolated from formalin-fixed paraffin-embedded samples. In this study, NGS was performed for one shortlisted ASD family (father, mother, affected child with autistic phenotype). According to Najmabadi et al. (2011) and Stankiewicz and

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Beaudet (2007), the introduction of NGS led to exponential increase of elucidated genetic causes in both extremely rare and common diseases and also provides linkage information in large extended families and detects de novo mutation or mosaicism in patients without any prediction about mutated gene.

A novel heterozygous missense mutation was identified at Chromosome 1, at 638(G>C) position in exon five (5) of RAS protein activator like-2 (RASAL2) gene. The (G>C) substitution changes the amino acid sequence from serine (S) to threonine (T) at position 213. According to Donovan et al. (2002), RASAL2 gene encodes a protein that contains the GAP related domain (GRD), a characteristic domain of GTPase activating proteins (GAPs). Similarly, Simanshu et al. (2017) reported that GAPs act as activators of Ras super family of small GTPase. GAPs function as molecular switches, continuing cycle between active form with GTP and inactive form with GDP bound (Donovan et al., 2002; Simanshu et al., 2017). To become activated, GDP has to exchange for GTP and activate downstream targets, called effectors, which in turn trigger cellular response (Donovan et al., 2002; Simanshu et al., 2017). In contrast to other signaling pathways, the Ras super family of small GTPase has a specific role in transmitting extracellular stimuli into neurons, by regulating many important cell functions such as gene expression patterns, cytoskeletal organization, cell growth and differentiation, and neuron development (Bloch-Gallego & Anderson, 2023; Guo et al., 2020; Reichova et al., 2018; Simanshu et al., 2017).

Previously, numerous studies investigated the role of GAPs in regulating the activity of RAS proteins (Donovan et al., 2002; Simanshu et al., 2017). According to Simanshu et al. (2017), overactive RAS have an important role in human disease. In our study, we also identified de novo mutation in RASAL2 gene encoded GTPase activating protein, belonging to the RAS family. Patients having mutated gene exhibit autistic phenotypes like improper social interaction and communication and repetitive behavior (Faras et al., 2010; Lord et al., 2020). According to The Human Protein Atlas, expression of RASAL2 gene is abundant in brain cells such as cerebral cortex, cerebellum, basal ganglia, and hippocampal formation. Hence, summary of all the literature discussed showed that RASAL2 gene is important factor in regulation

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of RAS signaling pathway (Guo et al., 2020; Reichova et al., 2018; Simanshu et al., 2017). According to Reichova et al. (2018), Autism Spectrum Disorder is associated with the regulation of spine plasticity and dendrite through Ras signaling pathways. Recently, studies identified that GTPase/Ras signaling is involved in autism spectrum disorders (Guo et al., 2020; Reichova et al., 2018). Similarly, Chiocchetti et al. (2015), Salcedo-Arellano et al. (2021), and Spina Nagy et al. (2021) showed that variation in synaptic proteins is linked with medical conditions in autistic patients. In an attempt to increase our understanding of the basic genetic causes of Autism Spectrum Disorder, Next Generation Sequencing was performed which identified a novel mutation (G>C) in RASAL2 gene in an autistic child that had not been previously reported in Pakistani population (Zhang et al., 2019; Ilyas et al., 2020; Najmabadi et al., 2011). Furthermore, the predicted protein structure of mutant RASAL2 gene suggested a potential role in cell signaling and subsequent disruption of cellular pathways, ultimately leading to the hallmark features of neurodevelopmental disorder (Parenti et al., 2020; Salcedo-Arellano et al., 2021; Reichova et al., 2018).

According to Guo et al. (2020), de novo mutation in RAC1 gene, an important Rho GTPase family member, has been identified in patients displaying hyperactive behavior and stereotypic movements. Similarly, Bloch-Gallego and Anderson (2023) investigated Rho GTPases in motor disorders associated with neurodevelopmental pathologies. The affected individual in this study shows diverse clinical manifestations including hyperactivity, repetitive behavior and social isolation, so it is hypothesized that the said mutation in RASAL2 gene is possibly responsible for NDDs in this family (Bloch-Gallego & Anderson, 2023; Guo et al., 2020).

By uncovering previously unknown genetic factors, our findings contribute to enhance the body of knowledge surrounding the molecular basis of neurodevelopmental disorders (Parenti et al., 2020; Ropers, 2008; Parenti et al., 2020). In addition to deepening our understanding of the disorder itself, this study also paves the way for targeted therapeutic interventions and genetic counseling for families affected by the disorder (Parenti et al., 2020; Savatt & Myers, 2021; Savatt &

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Myers, 2021).

Conclusion

The conclusion emphasizes that recent advances in Next Generation Sequencing and whole exome sequencing have significantly improved the diagnostic and genetic understanding of neurodevelopmental disorders, though these technologies present bioinformatics challenges due to the massive data they generate. Future research must prioritize experimental validation using cellular and animal models to confirm the functional impact of identified genetic variants, while expanding genomic studies to include larger and more diverse cohorts through collaborative research efforts. Ultimately, translating these findings into clinical applications is essential for developing diagnostic approaches that enable early detection and personalized treatment strategies for affected individuals and their families.

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