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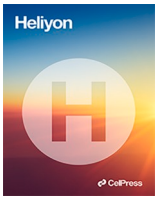

Research article

RNA-Seq data analysis reveals novel nonsense mutations in the NPR3 gene leading to the progression of intellectual disability disorder

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ABSTRACT

Intellectual disability (ID) is a progressive disorder that affects around 1–3% of the world's population. The heterogeneity of intellectual disability makes it difficult to diagnose as a complete disease. Genetic factors and major mutations play a noticeable role in the development and progression of ID. There is a high need to explore novel variants that may lead to new insights into the progressive aspects of ID. In the current course of study, 31 samples of ID from different studies available on GEO (GSE77742, GSE74263, GSE90682, GSE98476, GSE108887, GSE145710, and PRJEB21964) datasets were taken for the study. These datasets were analyzed for differential gene expression and single nucleotide polymorphism (SNPs). The SNPs of high impact were compared with the differentially expressed genes. Comparison leads to the identification of the priority gene ie NPR3 gene. The identified priority gene further was evaluated for the effect of the mutation using a Mutation Taster. Structure comparison analysis of the wild and mutated proteins of the NPR3 gene was further carried out by UCSF Chimera. Structural analysis reveals the anomalies in protein expression affecting the regulations of the NPR3 gene. These findings identified a novel nonsense mutation (E222*) in the downregulated NPR3 gene that leads to anomalies in the regulation of its protein expression. This missense mutation reveals a major role in causing ID. Our study concludes that the decrease in the expression of the NPR3 gene causes delayed sensory, motor, and physiological functions of the human brain leading to neurodevelopmental delay that causes ID.

1. Introduction

Intellectual disability (ID) disorder is an exponentially growing neurodevelopmental disorder that affects around 1–3% of the world population. According to the Diagnostic and Statistical Manual of Mental Disorders, 5th Edition (DSM-5), intellectual disability is a neurodevelopmental disorder that occurs during childhood leading to intellectual, social, conceptual, and practical difficulties [1]. The disorder is characterized by deficit adaptive behavior and impaired intellectual functioning [2]. Intellectual disability can be autosomal dominant [3], autosomal recessive [4], or X-linked [5]. The autosomal recessive form of ID may or may not produce any symptoms. The autosomal dominant ID is generally caused due to either copy number variations (CNVs) or heterozygous mutations in

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different reported genes [6]. X-linked ID is one of the most researched forms of ID. There are around 10–12 % of genes present on the X chromosome that have been linked to ID [7]. Intellectual disability is caused not only due to polygenes but environmental factors as well affecting a broad range of behavioral and clinical characteristics [8]. The heterogeneity of intellectual disability does not only change its frequency ratio worldwide [9] but also makes its diagnosis at an early stage challenging. With the advent and progression of next-generation sequencing, genes and mutations playing a key role in the development and progression of intellectual disability can be identified.

There are several attempts made for the identification of mutations that can cause intellectual disability using different techniques and approaches. Tham et al. identified 5 de novo heterozygous truncating mutations in gene KAT6A using a whole exome sequencing approach [10]. Lee et al. identified two recurrent recessive mutations (pT99 M and pE253K) in the motor domain of neuron-specific motor protein KIF1A as an important cause of cognitive impairment, axonal neuropathy, spastic paraparesis, and cerebellar atrophy [11]. Whole exome sequencing done by Nieh et al. also revealed de novo mutations in KIF1A as an important cause of brain atrophy and encephalopathy [12]. Baker et al. wrote a case series that identified de novo heterozygous missense mutations (M303K, D304G, D366E, I368T, and N371K) in the C2B domain of SYT1 gene [13]. Harripaul et al. reviewed the loss of function mutations in ABI2, MPDZ, MAPK8, SLAIN1, PIDD1, TRAPPC6B, TBC1D23, UBA7, and USP44 genes responsible for autosomal recessive intellectual disability along with missense mutations occurring in genes TET1 and BDNF [14]. According to the American College of Genetics

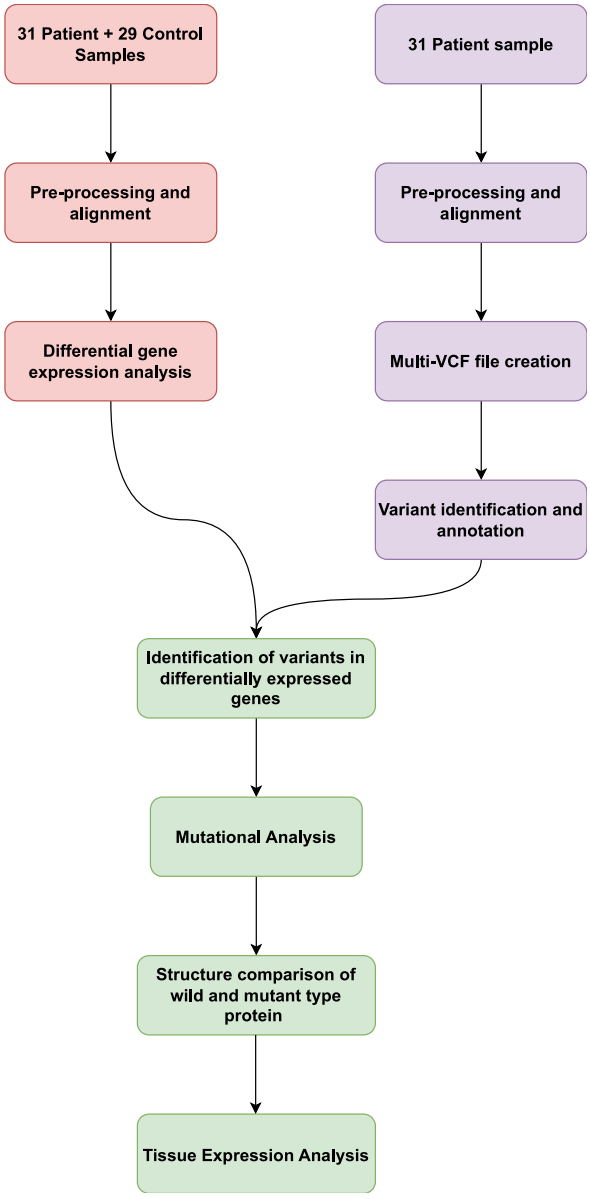


Fig. 1. The complete pipeline for variant identification.

(ACMG) [15], the homozygous mutation 554T > C in ACTL6B, 3666_3672 duplication in BRD4, 113A > G in DDX3X [16], 1918G > C in GRIN1, 12719C > T in HUWE1, 827C > T in PGAP3, 629C > G in RA11, 1252 T > G in RBFOX1, 2380G > A in SCN2A, 1258C > T in SMARCA2, and 281G > A in SOX4 as likely pathogenic in the case of intellectual disability. Moreover, deletion at position 2155 in ADNP [17], deletion at 1181 and 1182 position in AHDC1, 1443C > A in DNMT3A, deletion of range 572–575 in DYRK1A [18], 1411G > T in FRMPD4, deletion at 6331 position in MEDI3L [19], 2667 G > A in NEXM1F, duplication at 600 position in POGZ, deletion at 414 and 415 position in SATB2, duplication at 696 position in SATB2, deletion at 1499 and 1500 position in SCN2A, 2933G > A in SMARCA4, deletion at 4641 and 4642 position in SON, deletion at 3756 and 3760 position in TRIP12, deletion at 623 and 624 position in TUBB, and 583C > T in ZBTB18 [20] are recognized as the pathogenic variants in intellectual disability.

Next-generation sequencing (NGS) is a powerful tool that can sequence personal genomes to detect a large number of genetic variants. The advancing methods of NGS data analysis can help us predict pathogenic and novel mutations that can cause ID [21]. The combination approach of NGS and bioinformatics tools can not only help in the identification of novel genes but also in screening out candidate genes. In the past several years, NGS has efficiently backed up ID research by providing new strategies at the clinical level [22]. The exome and transcriptome analysis evaluates the protein-coding region which is the hub of 85 % of the disease-causing mutations. These approaches help in predicting genes that can be targeted in the future for the diagnosis of the disease, and to study its prevalence and prognosis.

The transcriptome data analysis can not only help identify variants but also the differentially expressed genes (DEGs) in the diseased state. These DEGs play an important role in the development and progression of the disease. The combined approach of sequencing techniques, bioinformatics, and functional analysis plays a great role in identifying new ID-causing genes.

Thus, in the present study, we aim to target the disease by identifying mutations occurring in differentially expressed genes that may play an important role in the development of the disease. 25 samples of intellectual disability from different studies available on GEO (GSE77742, GSE74263, GSE90682, GSE98476, GSE108887, GSE145710, and PRJEB21964) were taken for the study. The datasets were analyzed for differential gene expression and single nucleotide polymorphism (SNPs). The SNPs of high impact were compared with the differentially expressed genes. The identified priority gene was further evaluated for the effect of the mutation using a Mutation Taster. Structure comparison analysis of the wild and mutated proteins of the NPR3 gene was further carried out by UCSF Chimera. Structural analysis reveals the anomalies in protein expression affecting the regulations of the NPR3 gene. These findings identified a novel nonsense mutation (E222*) in the downregulated NPR3 gene that leads to anomalies in the regulation of its protein expression. This missense mutation reveals a major role in causing ID.

2. Methodology

An intensive literature study was done for the analysis of variants occurring in differentially expressed genes. The complete workflow opted for in the present study has been depicted in Fig. 1.

2.1. Data retrieval

In the current study, we used only those studies that were based on transcriptomic sequencing of patients, and control samples were taken for analytical consideration. The GEO [23], SRA [24], and BioProject [25] databases of the National Centre for Biotechnology Information (NCBI) were used as the major platform for data retrieval and collection. A total of 29 control and 31 patient sample were taken for the analysis in the present study.

2.2. Quality control, trimming, and alignment

The quality of each sample present in each study was checked using FASTQC [26]. FASTQC is an analysis tool that performs quality control checks on raw sequence data derived from high-throughput sequencing pipelines. The low-quality reads and duplicated sequences were removed from the sample sequence using the Trimmomatic tool [27]. The trimmomatic tool performs different trimming jobs for illumine paired-end and single-ended data. FASTQC and Trimmomatic were accessed from the GALAXY platform [28].

Each study of each sample was aligned to the reference genome of *Homo sapiens* (hg38) using HISAT2 [29]. HISAT2 is a fast and sensitive alignment program that is more than 50 times faster as compared to TopHat2 and requires a moderate amount of storage. It gives better alignment quality than the pre-existing alignment tools. After alignment, the counts per million (CPM) of each transcript mapped onto the reference genome were calculated using FeatureCounts [30]. FeatureCounts is a program written in C language and is a part of the Subread package that counts reads for genomic features in SAM/BAM files. The GALAXY platform was again used for access to this software.

2.3. Differential gene expression analysis

The genes that were differentially expressed in the patient sample as compared to the control sample were analyzed using the DESeq2 [31] package of R Studio [32]. DESeq2 is an analysis package that is designed to carry out normalization, visualization, and differentiation of high-dimensional count data. The log fold change calculations in DESeq2 are done by the use of the empirical Bayes technique.

2.4. Variant analysis

The trimmed FASTQ files were mapped to the reference human genome hg38 using the BWA-MEM2 algorithm [33]. The mapped reads were further subjected to postprocessing for use in variant calling software at the next step. The paired-end reads from all samples were filtered to retain only those pairs for which both forward and reverse reads have been mapped to the reference genome. The duplicated reads that may arise due to PCR overamplification of the genomic fragments were further removed from the final BAM file. The variants were identified in the mapped and post-processed sequence reads using the FreeBayes algorithm [34] to obtain a multisample VCF file. FreeBayes is based on the Bayesian genetic variant algorithm that helps predict SNPs and INDELs. The multisample VCF file was then split into multiallelic variant records and the file was normalized to represent indels in left alignment. The VCF file was further annotated using SnpEff eff [35] to determine the impact of the mutation on protein expression.

2.5. Mutation analysis

The pathogenicity and the type of mutations occurring in NPR3 (transcript id: NM_001204375.1) were evaluated using a mutation taster [36] and the SIFT tool [37]. These are web-based applications that help in predicting the impact of mutations on several biological processes.

2.6. Structural modeling and comparison

Structural analysis of variant E222* in NPR3 was performed based on the known X-ray structure of human natriuretic peptide receptor-C (PDB ID: 1YK0) which exhibits 100 % similarity with the mutated sequence. We modeled the structure of NPR3 variant E222* using SwissModel [38] and structure visualization and comparison were done using UCSF Chimera [39].

2.7. Tissue expression analysis

The expression of the NPR3 gene in different tissues of the human brain was studied in the human protein atlas [40]. The human protein atlas contains the expression summary at both mRNA and protein levels covering data from around 15,297 (78 %) protein-coding genes.

3. Results

3.1. Data retrieved

NCBI was browsed to select 7 studies related to transcriptomic sequencing of intellectual disability patients. In each study, all samples were taken for analysis. Studies that dealt with multiple conditions were further broken down into different groups for analysis. Table 1 lists the details of the studies selected for the analysis.

3.2. Pre-processing and alignment

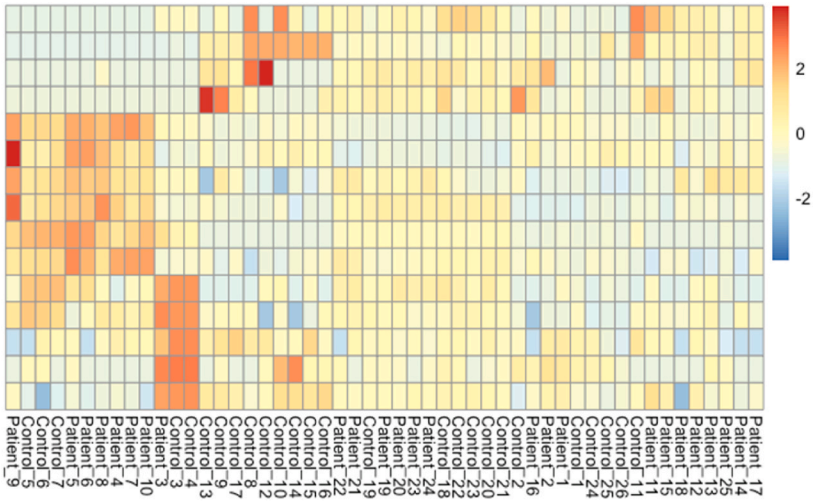
Each sample of all the studies was independently run through FASTQC to check the quality of the reads. The poor-quality reads and duplicated sequences were deleted. The alignment of all the sequences was done through the human genome (hg39, 2013 release). The aligned sequences were further used to count the expression of genes in each sample.

3.3. Differential gene expression analysis

The downregulated genes in the patients with intellectual disabilities were identified using DESeq2. Those genes that have an adjusted p-value less than 0.05 and log a 2-fold change of less than 0 were considered as the downregulated genes. DESeq2 identified 6 genes as downregulated in the patient sample namely NPR3, OVCH1-AS1, LSM14B, NOTCH1, ARV1, and ZNF75D. The differential expression analyzed through DESeq2 has been depicted in the form of a heatmap, MA plot, and volcano plot in Fig. 2.

Table 1
Details of the study included for analysis.

Accession ID	Number of Control samples	Number of Patient Samples	Cell/Tissue Type	Type of ID	Reference
GSE77742	2	1	Skin fibroblast	ADID	[41]
GSE74263	2	2	Lymphocytes	XID	
GSE90682	3	7	SK-N-SH Cell line	ARID	[42]
GSE98476	10	8	Immortalized lymphoblastoid cell line (LC)	Idiopathic ID	[43]
GSE108887	6	6	Blood	ARID	
PRJEB21964	3	1	Peripheral blood mononuclear cell	Idiopathic XID	
GSE145710	3	6	Transformed Lymphocyte Cell Line	XID	[44]



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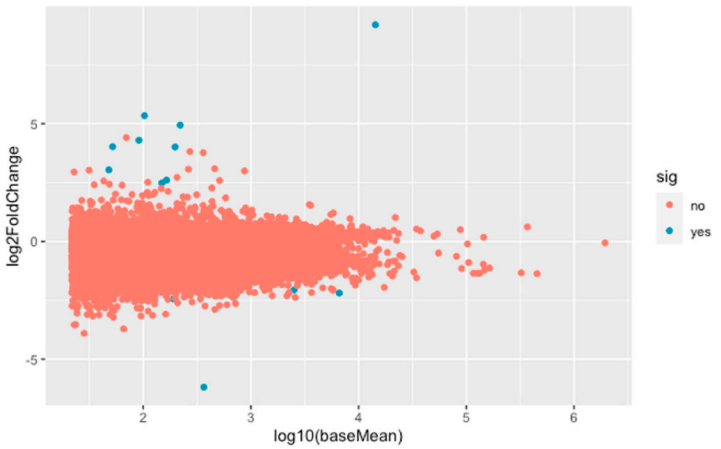

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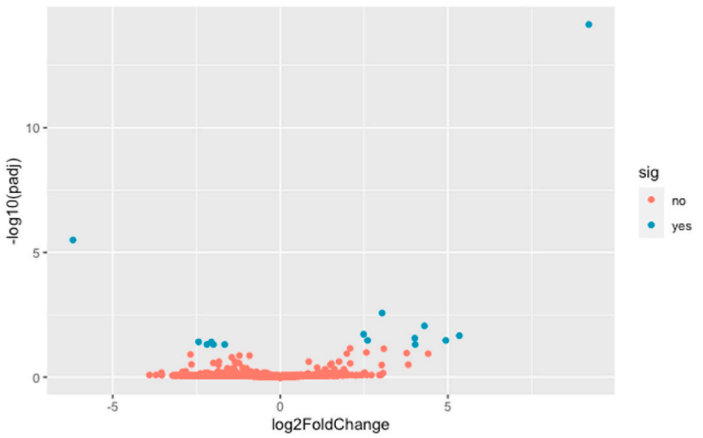

(c)

Fig. 2. (a) Heatmap showing the upregulated genes in red, downregulated genes in blue, and non-significant genes in yellow **(b)** MA Plot **(c)** Volcano plot obtained from DESeq2. Blue represents significantly expressed genes while red represents non-significant genes.

3.4. Variant analysis

The variant analysis performed using SnpEff eff identified a novel nonsense mutation occurring in the downregulated differentially expressed gene NPR3 having a high variant impact score 4. Fig. 3 shows the graphical representation of the mutations occurring in NPR3 gene.

3.5. Mutation analysis

The pathogenicity of the novel mutation (E222*) occurring in NPR3 was identified using mutation taster and SIFT. The nonsense mutation affects the functioning of the protein due to the sudden stop codon that disrupts the structure of the protein. The mutation occurs in the protein-coding region and acts as a deleterious variant. Table 2 enlists the SIFT and mutation taster predictions for E222* mutation in NPR3 gene.

3.6. Structure modeling and comparison

The structure of NPR3 variant E222* modeled using SwissModel and 1YK0 as the template is a monomer having the global mean quality estimate (GMQE) of 0.72, QMEANDisCo global score of 0.75 $\pm$ 0.07, and QMEAN Z-score of -2.06. The structures of the mutant NPR3 show 96.57 % similarity with the wild-type structure of NPR3. The structural comparison shows that the NPR3 structure shortens due to the nonsense mutation as shown in Fig. 4.

3.7. Tissue expression analysis

The expression of the NPR3 gene was seen in the brain tissue through the human protein atlas. The Tau specificity score of the gene in the human brain was 0.22 indicating identical expression of the gene across all cells and tissues. Fig. 5 illustrates the expression of NPR3 gene in different regions of the brain along with its nTPM values listed in Table 3.

4. Discussion

The study of genes involved in intellectual disability has increased over the past few years due to the development of advanced sophisticated sequencing techniques. The new massively parallel sequencing methods and different human variant databases can play an important role in the diagnosis of ID. In the present study, we identified NPR3 to be downregulated in 31 patients with intellectual disabilities. Furthermore, the variant analysis identified a novel nonsense mutation in the NPR3 gene which clearly explains its downregulation in the patient sample.

Natriuretic peptide receptor-C or NPR3 is a cell surface receptor present in the vasculature, adrenal, kidney, and brain. The major function of NPR-3 is to clear natriuretic peptides that play a key role in maintaining vascular tone and intravascular volume [45]. The family of natriuretic peptides (NP) binds to these receptors to elicit several renal, vascular, and endocrine effects. The key role of natriuretic peptides is well established in the human cardiac system by affecting processes like sodium and water excretion, renin-angiotensin-aldosterone system, and vasomotor tone [46]. These peptides can be degraded by mechanisms like clearance by NPR-3 and enzymatic degradation catalyzed by membrane metalloendopeptidase [47].

In recent times, the expression of the NPR3 gene has been studied in the human brain as well. The presence of NP and NPR has been detected in several brain tissues like cerebral vessels, neuronal structures, and astrocytes in the frontal lobe [48]. The results of the human protein atlas indicate sufficient expression of NPR3 protein in different brain regions like the cerebral cortex, hippocampal formation, amygdala, basal ganglia, thalamus, hypothalamus, midbrain, cerebellum, pons, medulla oblongata, spinal cord, and white matter. Different regions of the brain perform different functions. The cerebral cortex is involved in functions like reasoning, thought, emotions, memory, consciousness, and language [49]. The amygdala is the core of the neural system that processes fear and threatening stimuli [50]. The basal ganglia are involved in motor learning behaviors, executive functions, and emotions [51]. Midbrain is involved in processing auditory and visual information, and governing motor control [52]. The cerebellum plays a key role in regulating higher cognitive and emotional functions [53]. Pons are essential for vital body functions like sleep, respiration, eye movement, swallowing, facial movement, hearing, conscious maintenance, and posture [54]. The medulla oblongata is involved in the regulation of several autonomic, respiratory, and cardiovascular functions [55]. White matter is an essential brain component involved in the conduction of nerve impulses [56]. Thus, any change in the expression of proteins in the brain can alter the physiological and mental functions of the brain. This clearly explains the different neurological symptoms that appear in patients with intellectual

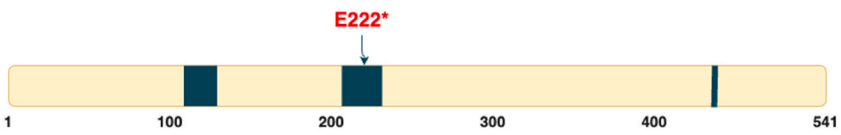

Fig. 3. E222* mutation occurring in NPR3 gene. Green areas represent disulfide bonds in the protein. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Results of E222* mutational analysis obtained from SIFT and mutation taster.

Chrom	Substitution	Region	SNP Type	SIFT Prediction	Mutation Taster Result	Mutation Type
5	E222*	Exon CDS	Nonsynonymous	Damaging due to stop	Deleterious	Nonsense mutation

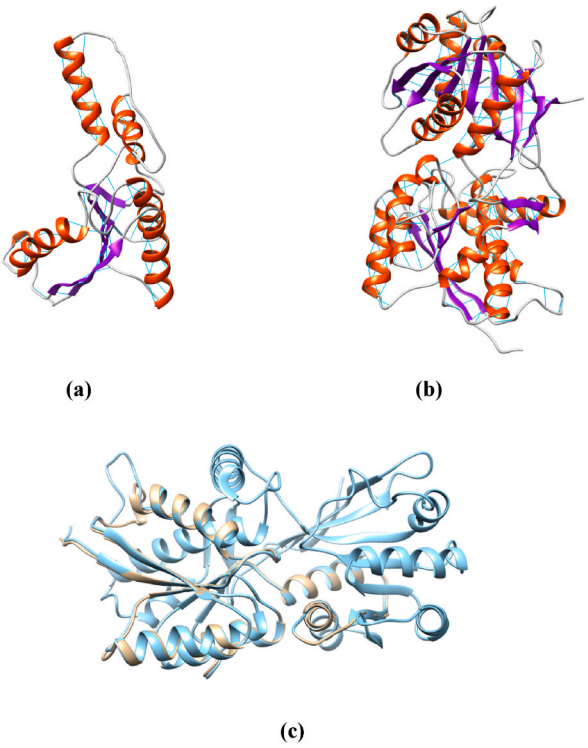

Fig. 4. **(a)** Mutated structure of NPR3 **(b)** Wild type structure of NPR3. The colors depict different secondary structures in the protein. Red: Helix, Purple: Strand, Grey: Coil. **(c)** Structural comparison of wild and mutant NPR3 where blue structure represents the wild NPR3 and grey structure represents mutated NPR3. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

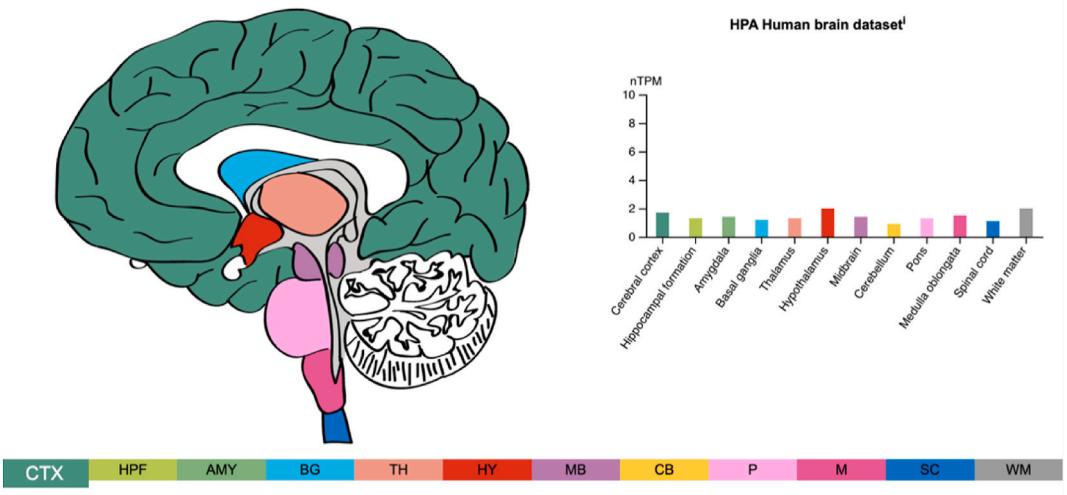

Fig. 5. The expression of the NPR3 gene in different brain tissues obtained from the human protein atlas.

Table 3
The expression value of NPR3 in different regions of the brain.

S.No.	Human Brain Region	nTPM (Transcripts per million)
1.	Cerebral cortex	1.7
2.	Hippocampal formation	1.3
3.	Amygdala	1.4
4.	Basal ganglia	1.2
5.	Thalamus	1.3
6.	Hypothalamus	2.0
7.	Midbrain	1.4
8.	Cerebellum	0.9
9.	Pons	1.3
10.	Medulla oblongata	1.5
11.	Spinal cord	1.1
12.	White matter	2.0

disabilities.

Similar results have been found by certain researchers working in the field of intellectual disability. Sheth et al., performed exome sequencing in 19 Indian patients with idiopathic neurodevelopmental disorders and found mutations in the NPR3 gene responsible for neurodevelopmental delay [57]. Devotta et al., investigated the important role of NPR3 gene in the formation of progenitors neural crest and cranial placode. The clearance and signaling function of NPR3 gene in the progenitor cell very well establishes its essential role in the development of fetal brain [58]. Several researchers have also reported the role of NPR3 gene in enhancing the growth and development of skeletal system in humans. This indicates the possibility of loss of function of NPR3 gene as the probable cause of skeletal phenotypic symptoms prevalent in patients with severe or profound ID [59]. However, in vitro research studies can give a better validation of the findings concluded in the present study.

Though the results are very convincing and have much impact on the findings of ID, the only limitation of the study lies that the available experimental data of ID from GEO is critically analyzed and well interpreted rather than own sequenced data. It is well established that *in silico* interpretations majorly give significant findings as this is the importance and cost effectiveness of computational experiments hence this limitation may also be overruled when results are in linearity with the study hypothesis.

5. Conclusion

Mutations can cause significant changes in the DNA sequence of the cell that may interrupt the normal expression of the protein in biological processes. The current study identified novel nonsense mutations in the NPR3 protein expressed in the brain. Nonsense mutations refer to the alteration of protein sequence in a way that causes the premature termination of the protein. The nonsense mutation causes a loss of function change by adding a premature stop to the protein-coding NPR3 gene leading it to a null allele [60]. Nonsense mutations are the main cause of 10 % of the genetic diseases [61]. In our study, the nonsense mutation occurring at position 222 of the NPR3 gene reflects a major cause for intellectual disability causation. The effect of the mutation on the structure of NPR3 proteins reveals the anomalies in protein expression affecting the regulations of the NPR3 gene. Our study concludes that the decrease in the expression of the NPR3 gene causes delayed sensory, motor, and physiological functions of the human brain leading to neurodevelopmental delay that causes intellectual disability. Several therapeutic approaches can be adopted to counteract nonsense mutations occurring in genes. These approaches include NMD inhibition, PTC readthrough, and screening of molecules that can correct nonsense mutation [62–65]. More studies need to be conducted to efficiently treat nonsense mutation.

The discovery of novel nonsense mutations in the NPR3 gene, identified through RNA-Seq data analysis, holds promise for advancing our understanding and management of intellectual disability disorder (IDD). These findings pave the way for further research into the functional consequences of these mutations, potentially shedding light on the molecular mechanisms underlying IDD progression. Moreover, the identification of these mutations offers opportunities for earlier diagnosis through genetic screening, facilitating personalized treatment approaches tailored to individual patients. Insights gained from studying these mutations may also lead to the development of new therapeutic targets, potentially improving outcomes for individuals with IDD. Additionally, this discovery may inspire broader research into the genetic basis of IDD and related neurological disorders, ultimately enhancing our ability to support affected individuals and families through genetic counseling and tailored interventions.

Thus, our study concludes that the decrease in the expression of the NPR3 gene in the patient sample can be an alarming sign of neurodevelopmental issues leading to delayed sensory, motor, and physiological functions of the human brain leading to neurodevelopmental delay and intellectual disability.

Data availability statement

The data used in the present study is available in the BioProject and GEO database of NCBI (www.ncbi.nlm.nih.gov). The accession ID of the data used is provided in the result section of the manuscript.

CRediT authorship contribution statement

Prekshi Garg: Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Farrukh Jamal:** Writing – review & editing, Supervision. **Prachi Srivastava:** Writing – review & editing, Visualization, Validation, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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