Research article

The investigation of nonsynonymous SNPs of human *SLC6A4* gene associated with depression: An *in silico* approach

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ABSTRACT

Genetic polymorphism of the *SLC6A4* gene is associated with several behavioral disorders, including depression. Since studying the total nonsynonymous single nucleotide polymorphisms (nsSNPs) of the *SLC6A4* gene at the population level is a difficult task, we aim to utilize *in silico* approach to detect the most deleterious nsSNPs of the *SLC6A4* gene. In our study, 7 computational tools were used in the initial stage, including SIFT, Polyphen-2, PROVEAN, SNAP2, PhD-SNP, PANTHER, and SNPs&GO to find out the most damaging nsSNPs. In the second phase, we performed structural, functional, and stability analysis of *SLC6A4* protein by popular computation tools, including I-Mutant 2.0 and MutPred2. Also, the ConSurf server was utilized to find the conserved region of the *SLC6A4* protein to determine the relationship between these conserved regions with high-risk nsSNPs. Based on these analyses, 5 high-risk mutations of the *SLC6A4* protein were selected. Then, we carried out comparative modeling by using the Robetta server and aligned the mutant protein model with the native protein structure. Later, we performed the post-translational modification and functional domain analysis of the *SLC6A4* protein. This study concludes that Arginine → Tryptophan at position 79 and Arginine → Cysteine at position 104 are the two significant mutations in *SLC6A4* protein which might play an essential role in causing diseases. Future studies should take these high-risk nsSNPs (rs1221448303, rs200953188) into consideration while exploring diseases related to the *SLC6A4* gene. Besides, our research is the first-ever comprehensive *in silico* investigation of the *SLC6A4* gene. Thus, the findings of this study could be beneficial for developing precision medicines against diseases caused by *SLC6A4* malfunction. Furthermore, extensive wet-lab research and experiments on various model organisms might be helpful to investigate the precise role of these damaging nsSNPs of the *SLC6A4* gene.

1. Introduction

Depression is a prevalent mental disorder all across the globe. According to the World Health Organization (WHO), more than 264 million people are affected with Depressive Disorders (DDs), and this pernicious trend of global health is continuing to rise [1]. The rate of depression varies from one continent to another. For instance, in the USA, the percentage of depressive disorder is 16.9% [2], while in the Asia Pacific, its ratio fluctuates from 1.1% to 19.9%. Research suggests that women suffer more from depressive disorders than men [3, 4]. Besides, depression has some deleterious and eye-opening consequences in human life.

For example, a study showed that 43% of people in the USA are negatively influenced by depression in their workplace and social communication. Moreover, depression inversely impacts productivity, and the global economy loses around 40 billion dollars per year [5, 6].

Among the several causes of depression, serotonin deficiency is the primary and earliest one [7]. The serotonin transporter, also called 5-Hydroxy Tryptamine Transporter (5-HTT), encodes by a gene called sodium-and-chloride-dependent solute carrier family 6 gene number 4 (*SLC6A4*). *SLC6A4* locates on chromosome 17 (from region 17q11.1 to 17q12) [8]. It acts as a modulator for 5-HTT since it stores back 5-HTT from the synaptic cleft to the presynaptic neurons, thus, facilitates the

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further use of 5-HTT. This event ensures the proper regulation of neurotransmitter concentration in the synaptic cleft [9, 10, 11]. Previous studies showed that polymorphism in the 5-HTTPLR region —a variable nucleotide tandem repeat (VNTR), methylation in the promoter and CpG island of the *SLC6A4* gene positively link up with various behavioral disorders, including depression [12, 13, 14, 15, 16, 17].

In recent times, *in silico* analysis plays a significant role in biology. It has a lot of biocomputing tools to analyze the structural and functional alteration of single amino acid. Besides, these tools allow the researcher to study the single nucleotide polymorphisms (SNPs), which is the most common (90%) genetic polymorphism of the human genome [18]. Also, the nonsynonymous SNPs (nsSNPs) are the single DNA sequence variation that can modify a protein's function by changing its amino acid sequence [19]. Several past studies showed the relationship between nsSNPs and complex human disorders, including hypertension, diabetes [20], polycystic kidney disorder [21], malignant melanoma [22], and cancer [23]. Previous research about the single nucleotide polymorphisms (SNPs) of the *SLC6A4* protein showed a positive relation with depression [24] and obsessive-compulsive disorder [25]. However, a comprehensive functional study about the total nsSNPs of the *SLC6A4* protein is yet to be done.

Therefore, we aim to conduct a computational analysis to predict the most pathogenic nsSNPs of the *SLC6A4* protein by utilizing various mutation-predicting tools and analyzing the functional domain, conservation, stability and structural alteration. Our study is the first comprehensive *in silico* investigation which covers the total nsSNPs of *SLC6A4* protein. Hence, our findings could benefit the precision medicine field by allowing disease treatment based on these genomic changes.

2. Materials and methods

The overall workflow for identifying and characterizing deleterious nsSNPs in the *SLC6A4* gene is shown in Figure 1.

2.1. Mining nsSNPs

A total of 360 nsSNPs related to the human *SLC6A4* gene were collected from the dbSNP database (https://www.ncbi.nlm.nih.gov/). We used UniProt database (https://www.uniprot.org/) to retrieve all the information related to the *SLC6A4* gene.

2.2. Prediction of the most damaging nsSNPs

We used various bioinformatics tools to determine the most deleterious and damaging nsSNPs. Those tools were: SIFT: Sorting Intolerant From Tolerant, PolyPhen-2: Polymorphism Phenotyping v2, PROVEAN: Protein Variation Effect Analyzer, PANTHER: Protein Analysis Through Evolutionary Relationships, PhD-SNP: Predictor of human Deleterious Single Nucleotide Polymorphisms, SNAP2: Screening for Nonacceptable Polymorphisms 2 and SNPs&GO.

SIFT and Polyphen-2 have been widely used bioinformatics tools to predict pathogenic nsSNPs. SIFT predicts whether substitution for the amino acid sequence is tolerated or deleterious for protein. If the SIFT score for a query position is less than 0.05, then it predicts as deleterious or intolerant. However, if the SIFT score for a query position is equal to or greater than 0.05, then it indicates as tolerated [26]. Polyphen-2 predicts the possible effect of each substitution on the structural and functional properties of a protein. This prediction comes from the structural and evolutionary conservation analysis. Unlike SIFT, Polyphen-2 usually makes three types of prediction: 'Probably Damaging,' 'Possibly Damaging' and 'Benign.' The range of score for Polyphen-2 varies between 0 to 1 [27]. Among the other five tools we utilized, PROVEAN predicts whether the variants functionally effective or not. Threshold value $\leq$ -2.5 provides the signal of deleterious nsSNP [28]. PANTHER predicts whether the particular substitution is pathogenic or not. The evolutionary conservation process measures it. Here, the conservation length of time determines the functional consequence of the protein [29]. PhD-SNP predicts outcomes based on the reliability index (RI) score. The results come in two forms: 'neutral' and 'disease' [30]. Neural network-based tool SNAP2 predicts the effect of a possible substitution on protein function. Score range of SNAP2 is varied from -100 (strong neutral) to +100 (strong effect) [31]. SNPs&GO predicts results based on a server called support vector machine (SVM), and it indicates that whether a certain substitution position of a protein is disease-associated or not. Its accuracy close to 82%, and a probability score >0.5 indicates probable mutation on parental protein structure [32].

2.3. Prediction of structural and functional properties

We used MutPred2 to find out the structural and functional properties of protein structure. MutPred2 (http://mutpred.mutdb.org/) web server
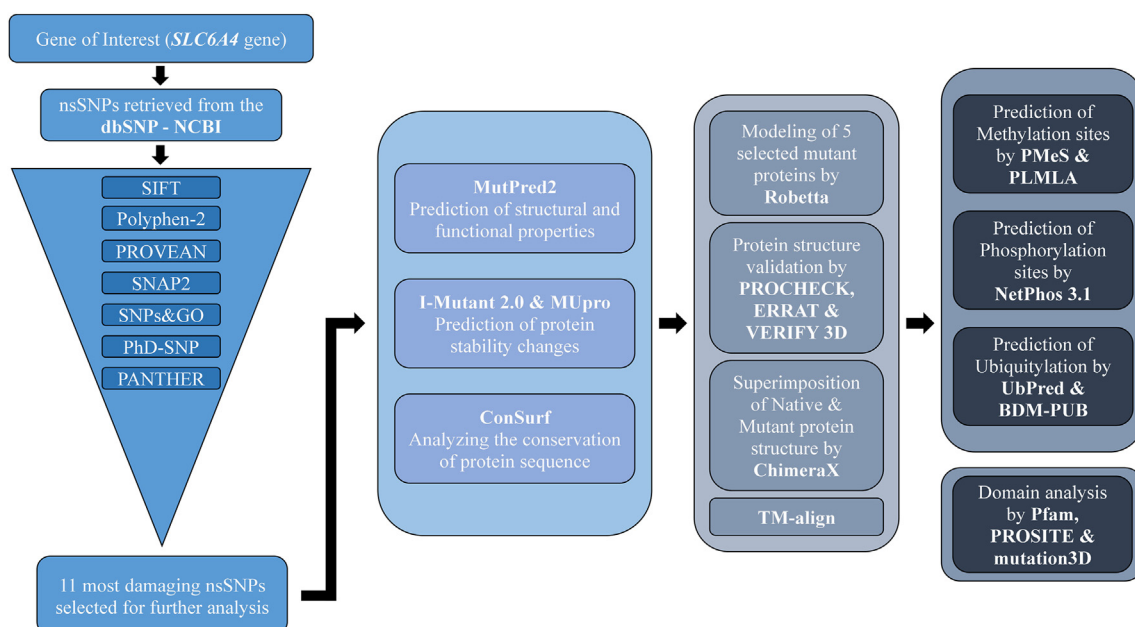

Figure 1. Overall workflow of identifying deleterious nsSNPs of the *SLC6A4* gene.

is developed to categorize amino acid substitutions as pathogenic or benign in humans. It also predicts their effect on more than 50 different protein properties, allowing for the assumption of pathogenic molecular mechanisms. It also aids in determining the disease's molecular cause [33].

2.4. Prediction of protein stability change

To determine the changes in protein stability, we used two different tools: I-Mutant 2.0 and MUpro. I-Mutant 2.0 (https://folding.biofold. org/i-mutant/i-mutant2.0.html) is a neural network-based web server. We used it to predict any change in the stability of protein after getting mutated. It predicts results using a reliability index (RI) ranging from 0 to 10, with 10 being the most reliable. It also assesses the degree of protein destabilization and the free energy change value (DDG) with the scale indicating whether it will increase or decrease. A DDG value less than 0 indicates a decrease in protein stability, whereas a DDG value greater than 0 indicates an improvement in protein stability [34].

The MUpro (http://mupro.proteomics.ics.uci.edu/) server was also commonly used to assess protein stability. Support Vector Machines (SVM) and Neural Networks are two machine learning methods used to create this web server. These methods estimate the impact of single-site amino acid mutations on protein stability and report the results as an increase or decrease based on a positive or negative score [35].

2.5. Analyzing the conservation of protein sequence

ConSurf (https://consurf.tau.ac.il/) is a web server that uses protein sequence to estimate evolutionary conservation of amino acid positions. The analysis utilizes phylogenetic relationships between homologous sequences to determine a protein's functional regions by assessing the degree of conservation. The scale, which range from 1 to 9, reflects the degree of amino acid conservation over time. Grade 9 and grade 1 corresponds to the conserved and variable, respectively [36].

2.6. Comparative 3D protein modeling

The wild type structure of our *SLC6A4* protein (PDB ID: 6W2B) was collected from RCSB PDB (https://www.rcsb.org/). From the UniProt server (https://www.uniprot.org/), we retrieved the FASTA sequence of the *SLC6A4* protein. Then, for modeling of the mutated protein structure, we modified the wild type sequence according to our selected top mutations. After that, we used the Robetta server (http://robetta.bakerlab.o rg/) to build the 3D structure of the 5 mutated proteins. The Robetta server uses a comparative modeling approach when the known structure is available. On the other hand, Robetta uses the de novo approach when the known structure is not found [37]. Robetta generated model 1 for every mutated protein was selected for validation (Figure 2). Validation was performed by using the SAVES v6.0 server (https://saves.mbi.ucla.
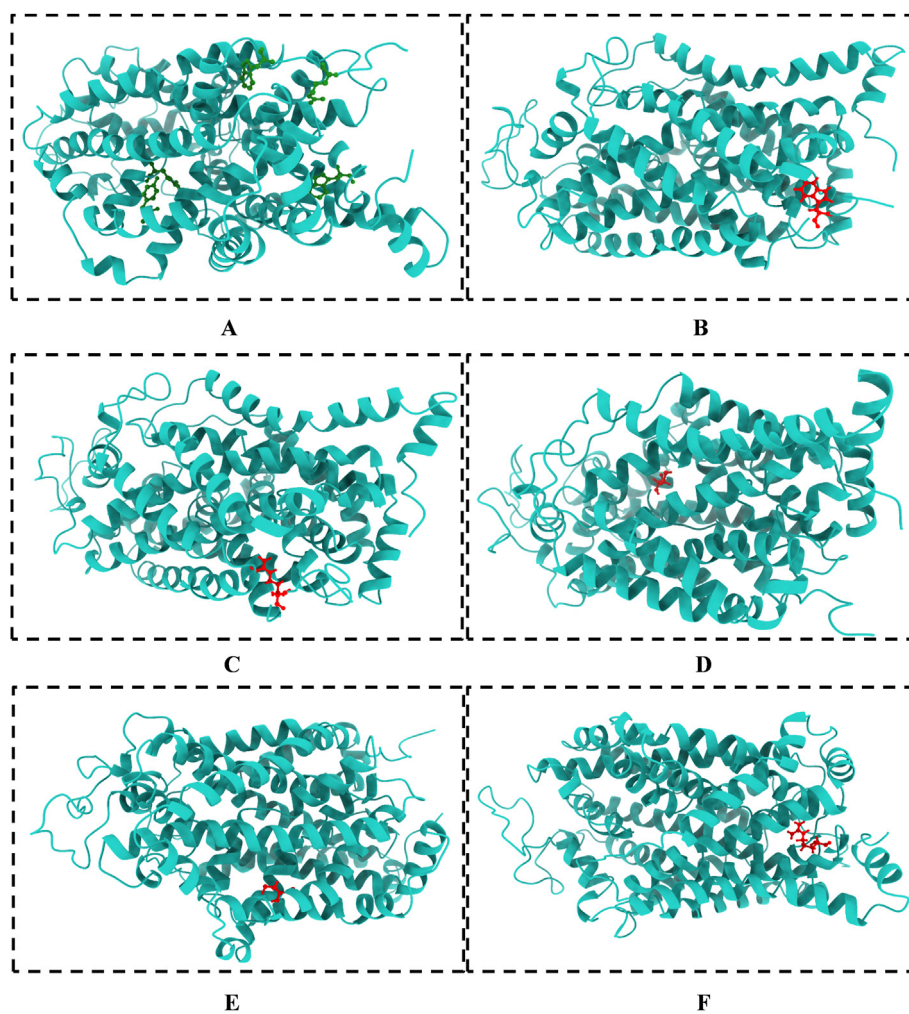

Figure 2. Native and predicted mutant structure of the *SLC6A4* protein. (A) Native protein structure, (B) Modelled mutant structure for R79W substitution, (C) Modelled mutant structure for W82R substitution, (D) Modelled mutant structure for R104C substitution, (E) Modelled mutant structure for Y121C substitution, (F) Modelled mutant structure for W151R substitution.

edu/). SAVES v6.0 use five different validation tools. Among them, we utilized ERRAT [38], VERIFY 3D [39] and PROCHECK [40] to validate our modeled mutant structures. Later, structural analysis between wild type protein and mutant models was carried out by ChimeraX software [41] and TM-align server (https://zhanglab.ccmb.med.umich.edu/TM-align/) [42].

2.7. Prediction of post-translational modification (PTM) sites

Probable methylation sites of the *SLC6A4* protein were predicted by two bioinformatics tools: PMeS and PLMLA. In PMeS, a higher SVMs score signals a better chance of either lysine or arginine getting methylated. PLMLA measures physiological properties around lysine residue based on grouped weight and position weight compositions of an amino acid sequence. This tool determines both methylation and acetylation roles around lysine residue [43, 44]. Like methylation, phosphorylation sites of the *SLC6A4* protein were predicted using a neural network-based tool: NetPhos 3.1, where a threshold score of more than 0.5 indicates a particular position gets phosphorylated [45]. Lastly, for the prediction of ubiquitylation, we utilized UbPred and BDM-PUB tools. In UbPred, a score of 0.62 or more indicates a particular position gets ubiquitinated. For BDM-PUB, we maintained the balanced cut off option to perform ubiquitylation analysis [46, 47].

2.8. Domain analysis of SLC6A4

To find the functional domain of the *SLC6A4* protein, we utilized Pfam [48] and PROSITE web server (https://prosite.expasy.org/). Pfam uses multiple sequence alignment and hidden Markov models to predict domains of a protein, while PROSITE uses structural and functional information. Later, we used mutation3D [49] web server to confirm the high-risk mutation positions in the particular domain of the *SLC6A4* protein.

3. Results and discussion

3.1. Dataset of interest

SNPs of *SLC6A4* gene were retrieved from the dbSNP database as it is the most widely used and comprehensive database [50]. The total number of SNPs was 10593. Among these, 360 were nsSNPs, 198 were synonymous, 892 occurred in 3 prime UTR, 99 in 5 prime UTR, 8572 in intron region, and remaining were other types (Figure 3). Only nsSNPs were selected for this study (S1 Table).
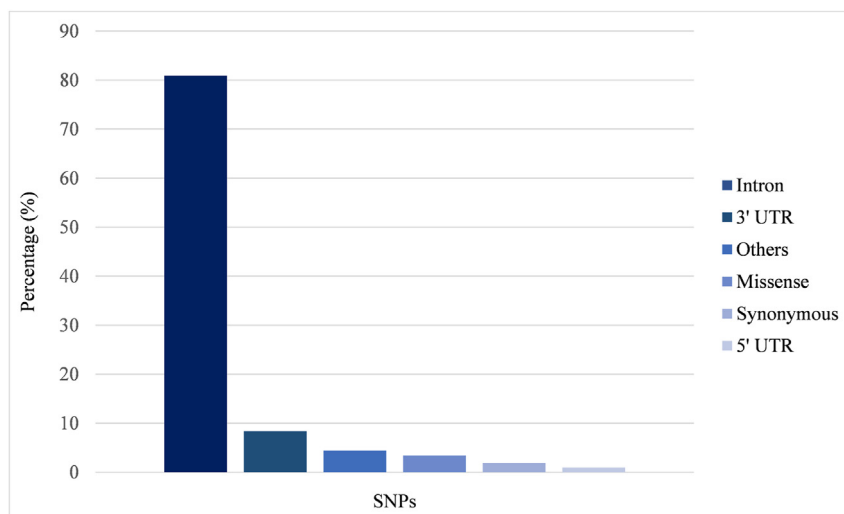
3.2. Deleterious nsSNPs prediction by in silico approach

In total, seven computational tools —SIFT, PolyPhen-2, PANTHER, PROVEAN, PhD-SNP, SNAP2, and SNPs&GO — were used to investigate all nsSNPs for evaluation of their effect on the structure and function of the *SLC6A4* protein.

According to SIFT scores, nsSNPs were classified as damaging for a tolerance index (TI) score of ≤0.05. Out of 360 nsSNPs, we found 176 nsSNPs as deleterious with a score of ≤0.05. The most damaging nsSNPs were predicted as probably damaging with a score of 1 by PolyPhen-2. Among 360 nsSNPs, 147 were predicted by Polyphen-2 as probably damaging. PANTHER on a scale of ≥0.5 predicted deleterious nsSNPs. Here, greater value leads to the more deleterious nature of nsSNPs. In total, 125 nsSNPs were predicted as a disease according to the PANTHER result. When the score was below 2.5, the PROVEAN tool predicted nsSNPs as deleterious. On the other hand, a score of >2.5 predicted as neutral. In total, PROVEAN predicted 190 nsSNPs as deleterious. Like other tools, the PhD-SNP predicted 169 nsSNPs as a disease based on a probability score higher than 0.5. And, the SNAP2 predicted 184 nsSNPs as deleterious, while SNPs&GO predicted 139 nsSNPs as diseased. Deleterious nsSNPs predictions by all seven tools are shown in Figure 4.

PANTHER and SNPs&GO predicted lesser number of nsSNPs as deleterious. However, all results were closely similar. So, we considered all the tools we used for our first step of sorting. Total results (predictions and scores) from all 7 mutation predicting tools are given in S2 table (S2 Table). The nsSNPs, which were predicted deleterious or damaging by all seven tools, were sorted out. 28 nsSNPs showed more damage with a score of 0 and 1 by SIFT and PolyPhen-2 (both HumDiv & HumVar), respectively. Among these, 11 consensus nsSNPs were sorted out based on PROVEAN and SNAP2 for further investigation (Table 1). These nsSNPs are: rs200953188, rs201480140, rs750079448, rs751233939, rs758510581, rs760517433, rs772080063, rs774597104, rs1221448 303, rs1245951483, rs1476888015.

3.3. Functional and structural alteration of SLC6A4 predicted by MutPred2

The top listed 11 nsSNPs of the *SLC6A4* protein were submitted to the MutPred2 server, and it predicted the probability scores and general score for each nsSNP. The alteration of properties for all 11 nsSNPs also predicted by the MutPred2 server. It includes loss and gain of allosteric site, strand, catalytic site, and helix with several other alterations (S3 Table). A previous study showed that MutPred2 capable of indicating the pathogenicity of missense variants [51]. Therefore, we can conclude that
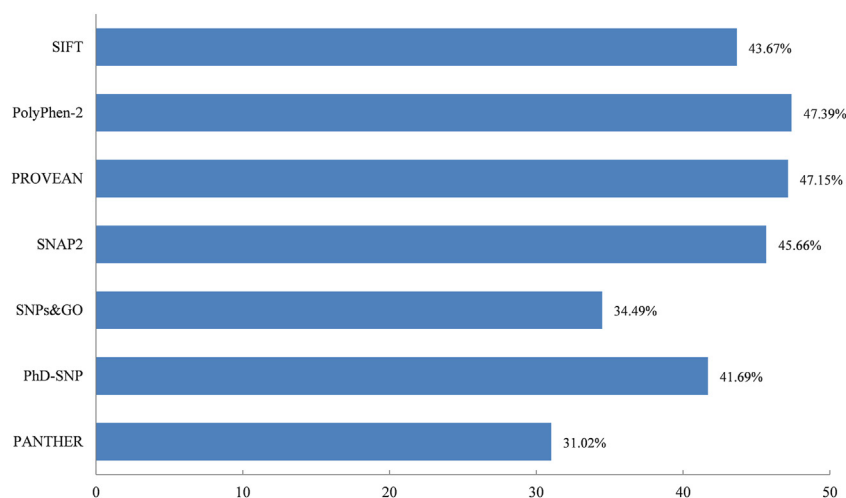

Figure 3. Distribution of SNPs in the *SLC6A4* gene. Intron = 80.92%, 3′ UTR = 8.42%, 5′ UTR = 0.93%, Missense/Nonsynonymous = 3.40%, Synonymous = 1.87%, Others = 4.45%.

Figure 4. Percentage of deleterious nsSNPs predicted by seven *in silico* tools. The deleterious nsSNPs identified by seven *in silico* tools: SIFT, Polyphen-2, PROVEAN, SNAP2, SNPs&GO, PhD-SNP, and PANTHER.

Table 1. Top 11 deleterious nsSNPs predicted by seven *in silico* tools.

Substitution	SIFT		Polyphen-2		PROVEAN		Phd-SNP		PANTHER		SNPs&GO		SNAP2	
	Pre	Score	Pre	Score	Pre	Score	Pre	RI	Pre	RI	Pre	RI	Pre	Score
R104C	DAM	0	PRO DAM	1	Del	-7.253	Dis	8	Dis	10	Dis	8	Effect	56
P131L	DAM	0	PRO DAM	1	Del	-9.228	Dis	9	Dis	10	Dis	7	Effect	85
T448M	DAM	0	PRO DAM	1	Del	-5.642	Dis	5	Dis	10	Dis	7	Effect	73
R234C	DAM	0	PRO DAM	1	Del	-7.728	Dis	7	Dis	7	Dis	7	Effect	52
Y121C	DAM	0	PRO DAM	1	Del	-8.21	Dis	9	Dis	9	Dis	8	Effect	51
R79L	DAM	0	PRO DAM	1	Del	-5.377	Dis	8	Dis	10	Dis	8	Effect	77
W82R	DAM	0	PRO DAM	1	Del	-11.893	Dis	8	Dis	10	Dis	6	Effect	92
W151R	DAM	0	PRO DAM	1	Del	-12.919	Dis	8	Dis	9	Dis	6	Effect	72
R79W	DAM	0	PRO DAM	1	Del	-6.431	Dis	8	Dis	10	Dis	7	Effect	85
C258R	DAM	0	PRO DAM	1	Del	-11.582	Dis	9	Dis	5	Dis	8	Effect	71
P590L	DAM	0	PRO DAM	1	Del	-8.508	Dis	8	Dis	8	Dis	5	Effect	70

Pre = Prediction, DAM = Damaging, PRO DAM = Probably damaging, Del = Deleterious, Dis = Disease.

several nsSNPs might cause structural and functional modification with the pathogenicity of *SLC6A4* protein.

3.4. Stability analysis of SLC6A4 by I-Mutant 2.0 and MUpro

To predict the stability of our *SLC6A4* protein, we submitted our selected 11 nsSNPs to both the I-Mutant 2.0 and MUpro server. According to the I-Mutant 2.0 result, all these nsSNPs correspond to decreased protein stability with a reliability index (RI) ranging from 1 to 8. Nevertheless, the MUpro server's result showed that P131L, R79L, T448M, P590L, and C258R variants increased the *SLC6A4* protein stability.

Previous studies showed that degradation, accumulation, and improper folding are also possible due to the destabilization of a protein [52, 53, 54]. Therefore, we can predict that these missense variants could change our protein's stability and maximize its functional damage.

3.5. Conservation profile of SLC6A4 predicted by ConSurf server

Evolutionary information is crucial for detecting mutation in a protein which might be associated with human health and diseases [55]. We used the ConSurf server to calculate the evolutionary conservation of amino acid residues of the *SLC6A4* protein and investigate the possible effects of our 11 top deleterious nsSNPs. According to the result, ConSurf predicted R104, P131, Y121, R79, W82, T448, P590, W151, C258 as

highly conserved with conservation scores between 7 to 9. One residue R234 of the top 11 is predicted as moderately conserved with a conservation score of 4 (S4 Fig.). Research showed that crucial amino acids, which actively participates in various biological mechanisms, is highly significant. These all are located in a protein's conserved region [56, 57]. Hence, we can conclude that nsSNPs, which are highly conserved, are immensely deleterious for both structural and functional properties of the *SLC6A4* protein.

Based on the analysis of MutPred2, MUpro, and I-Mutant 2.0, six variants showed possible structural and functional damage. Besides, they can also decrease the stability of our protein structure. However, 5 variants out of 6 are highly conserved, thus, selected for modeling (Tables 2 and 3).

3.6. Comparative modeling of native SLC6A4 and its mutants

Robetta generated models were validated using ERRAT, VERIFY 3D, and PROCHECK. ERRAT compared the models to a database of reliable high-resolution structures based on non-bonded atom-atom interactions. A high-resolution structure generally provides a 95% or higher overall quality factor [38]. In our study, all models generated more than 97% overall quality factor. VERIFY 3D compares the 3D profile of residues in structure. When more than 80% residue profile score ≥0.2 is considered as pass [39]. All our modeled structures passed in VERIFY 3D. PROCHECK generates a Ramachandran plot which is based on residues in most favoured regions. Higher than 90% residues in most preferred

Table 2. Structural, functional and stability analysis of the *SLC6A4* protein.

SNP ID	Variant	MUpro			I-Mutant				MutPred
		Prediction SVM	Score	Prediction NN	Score	Prediction	RI	DDG	P Value
rs200953188	R104C	Decrease	-0.886	Decrease	-0.546	Decrease	7	-1.48	0.942
rs758510581	Y121C	Decrease	-0.900	Decrease	-0.568	Decrease	2	0.43	0.95
rs772080063	W82R	Decrease	-1.054	Decrease	-0.559	Decrease	7	-1.69	0.95
rs1221448303	R79W	Decrease	-0.398	Decrease	-0.600	Decrease	7	-0.19	0.87
rs774597104	W151R	Decrease	-1.877	Decrease	-0.914	Decrease	9	-1.53	0.957

Table 3. Conservation score and prediction of top 5 nsSNPs.

SNP ID	Variant	ConSurf	
		Score	Prediction
rs200953188	R104C	9	Highly conserved and exposed(f)
rs758510581	Y121C	9	Highly conserved and buried(s)
rs772080063	W82R	9	Highly conserved and exposed(f)
rs1221448303	R79W	9	Highly conserved and exposed(f)
rs774597104	W151R	7	Buried(b)

(f): Predicted functional residue, (s): Predicted structural residue, (b): Predicted buried residue.

regions are considered as a good quality model [40]. PROCHECK result for our modeled structures was more than 90%. Results of structural validation are shown in Table 4. Then, these five mutant models were compared with native protein structures (6W2B). The superimposition between native and mutant protein structure was performed by ChimeraX software (Figure 5).

Superimposition between two models provides root-mean-square deviation (RMSD), which means the deviation of the two compared models. The higher RMSD value indicates a more significant deviation. TM-score also evaluates the structural similarity between two models ranging from 0 to 1, where 1 denotes fully similar and lower value means dissimilarity. The structure for R104C and Y121C mutations showed higher RMSD. Besides, W82R and R104C have lower TM-score. The RMSD and TM-score of the remaining mutations were nearly similar (Table 5). Similar studies have done many genes and proteins like the human *LEP* gene [58] and *TAGAP* protein [59].

3.7. Post translational modification (PTM) prediction of SLC6A4

Previous studies showed that PTM is highly significant in regulating protein structure and function. Disease like diabetes and circadian dysregulation have a positive association with PTM [60, 61]. We used several *in silico* tools to predict the PTM sites (methylation, phosphorylation and ubiquitylation) in the *SLC6A4* protein.

3.7.1. Methylation

Histone modification occurs through methylation of a lysine residue and modifies the gene expression. The PLMLA tool predicted 16 lysine residue sites that can get methylated. According to our ConSurf prediction, L29 and K279 are highly conserved. On the other hand, PMeS predicted only 4 methylated sites (S5 Table). Interestingly, out of four, three of them predicted as highly conserved (R79, R104, R298) by the

ConSurf server (Figure 6). Besides, we found that position 79 and 104 are the most deleterious nsSNPs sites for *SLC6A4* protein, where arginine substituted by tryptophan and cysteine, respectively.

Previously, Kim *et al.* showed that methylation in the promoter region of the *SLC6A4* gene is responsible for post-stroke depression [17]. Therefore, we can presume that these highly conserved methylation sites could significantly alter the *SLC6A4* gene expression and play a vital role in causing diseases.

3.7.2. Phosphorylation

Phosphorylation of a protein can lead to several results, e.g., activation or deactivation of a signaling pathway and altering a protein's structure and function [62, 63, 64]. In our study, probable phosphorylation sites in the *SLC6A4* protein predicted by NetPhos 3.1 server. In total, we found 60 phosphorylation sites (S5 Table). Among them, five are located in the highly conserved region (according to the prediction of the ConSurf server): R79, R104, F133, A285, and D452 (Figure 6). Interestingly, we previously found 79 and 104 positions as high-risk, deleterious nsSNP for the *SLC6A4* gene.

A past study showed that tyrosine phosphorylation directly links with behavioral disorders, including depression [65]. Hence, we can predict that these phosphorylation sites could enhance the chance of structural and functional alteration and could damage the significant function of the *SLC6A4* protein.

3.7.3. Ubiquitylation

Research showed that ubiquitylation is one of the reasons for protein degradation [66]. In our study, putative ubiquitylation sites in *SLC6A4* were predicted by UbPred and BDM-PUB tools. UbPred predicted only three ubiquitylation sites, while BDM-PUB predicted ten putative sites in *SLC6A4* protein (S5 Table). Three positions were common between UbPred and BDM-PUB predictions: 10, 29, and 37 (Figure 6). Among

Table 4. Validation of mutant structures of the *SLC6A4* protein.

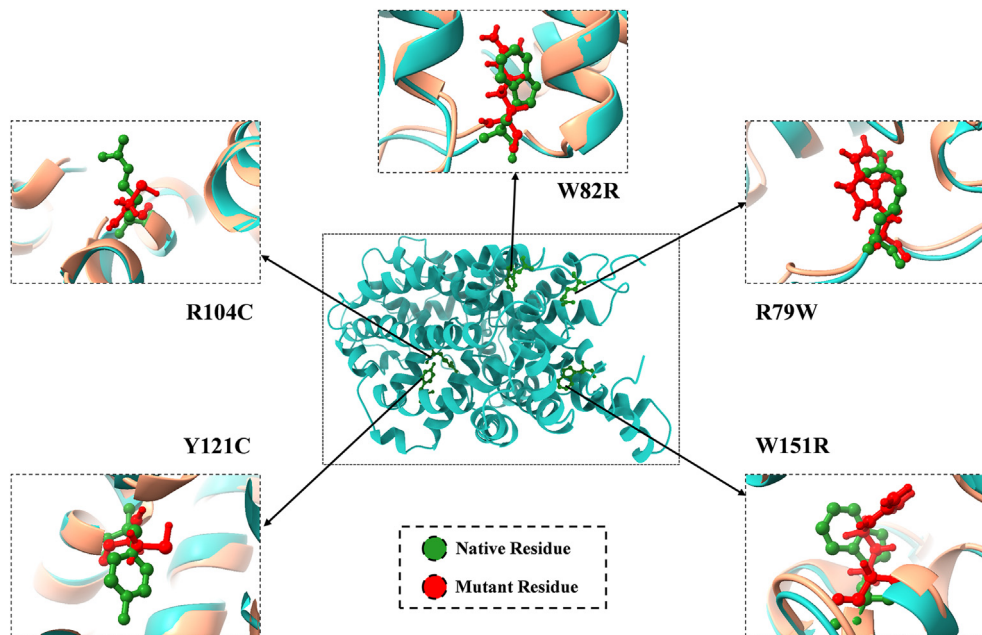| Mutation	ERRAT	VERIFY 3D	PROCHECK
R104C	98.8785	Pass	94.80%
Y121C	97.9439	Pass	95.40%
W82R	97.9478	Pass	95%
R79W	97.5746	Pass	95%
W151R	98.5047	Pass	95.40%

Figure 5. Comparison between native protein structure and its mutant forms. R79W: wild type Arginine substituted by Tryptophan at position 79, W82R: wild type Tryptophan substituted by Arginine at position 82, R104C: wild type Arginine substituted by Cysteine at position 104, Y121C: wild type Tyrosine substituted by Cysteine at position 121, W151R: wild type Tryptophan substituted by Arginine at position 151.

Table 5. Structural comparison between native and mutant structures by using ChimeraX and TM-align.

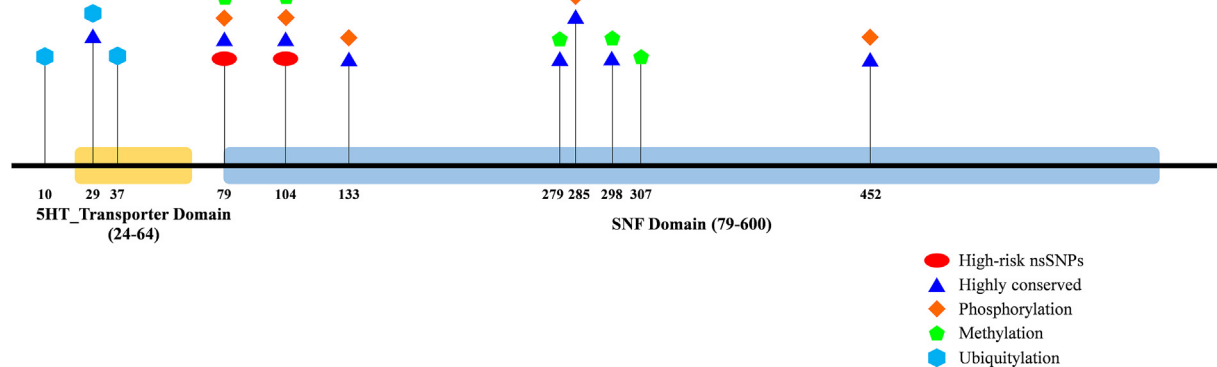| SNP ID	Mutation	RMSD	TM-align
rs200953188	R104C	0.846	0.98655
rs758510581	Y121C	0.855	0.98696
rs772080063	W82R	0.844	0.98652
rs1221448303	R79W	0.844	0.98719
rs774597104	W151R	0.84	0.98657

Figure 6. Schematic representation of the high-risk nsSNPs and their putative PTM sites of the *SLC6A4* protein. The figure illustrated the putative PTM sites of the *SLC6A4* protein and their relation with high-risk nsSNPs. Three types of putative PTM (methylation, phosphorylation, and ubiquitylation) sites predicted in both R79 and R104 positions. These two positions present in the SNF domain of the *SLC6A4* protein. Except for K10, K37 and R307, all positions are highly conserved.

these three positions, only K29 is highly conserved (according to the ConSurf prediction server).

A study by the Li *et al.* revealed that the ubiquitin proteosome system could change synaptic plasticity and play a critical role in the biology of long-term depression [67]. Therefore, like methylation and phosphorylation sites in the *SLC6A4* protein, these ubiquitylation sites can play an essential role in the degradation of the *SLC6A4* protein.

All methylation, phosphorylation, and ubiquitylation sites of the *SLC6A4* protein have not been fully reported yet. Therefore, these predicted PTM sites (which are highly conserved) could change the structural, functional, and biological role of the *SLC6A4* protein. Besides, these sites can decrease the stability of the *SLC6A4* protein. However, an opposite result could also possible since these PTM sites could neutralize the deleterious effects of high-risk nsSNPs.

3.8. Domain analysis of SLC6A4

In our study, Pfam, PROSITE, and mutation3D web server predicted the functional domain of the *SLC6A4* protein. Pfam and PROSITE predicted 5HT_Transporter domain (from amino acid 24 to 64) and Sodium: Neurotransmitter symporter Family (SNF) domain (from amino acid 79 to 600) (Figure 6). Later, mutation3D confirmed that our high-risk nsSNPs, rs1221448303 and rs200953188, are located in the SNF domain.

Previous research showed that mutation in the SNF superfamily of the *SLC6A4* gene is associated with behavioral disorders, including depression. Besides, the researcher often targets this superfamily to develop drugs against depressive disorders [68, 69]. Therefore, we can predict that R79W and R104C might be the potential substitution sites for developing precision medicine against diseases.

This research has some limitations, as we have only performed *in silico* analysis. A large-scale population study associated with nsSNPs of the *SLC6A4* gene in different parts of the world and wet-lab experiments in multiple model organisms might provide more robust verification of the findings of this *in silico* investigation. However, for *in vivo* experiments, this research can provide a solid base and facilitate researchers to narrow down their experimental hassles in wet-lab.

4. Conclusion

In this study, *in silico* analysis was performed to investigate the possible effects of nsSNPs on the structure, function, and stability of the *SLC6A4* protein. The five mutations (R104C, Y121C, W82R, R79W, and W151R) were found as the probable reason behind the structural and functional alteration of the *SLC6A4* protein, and probably modify its activity. Among the five mutations, Arginine → Tryptophan at position 79 and Arginine → Cysteine at position 104 were the two major mutations, as these two positions are highly conserved and putative PTM sites. Also, R79W and R104C are present in the SNF domain of the human *SLC6A4* protein, which often targets to develop drugs against behavioral disorders. Therefore, these nsSNPs could be the potential choice regarding the structural and functional alteration of the *SLC6A4* protein and its associated diseases. Comprehensive population-based study and wet-lab experiments in multiple model organisms may help to determine further prove.

Declarations

Author contribution statement

Md. Amit Hasan: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Fuad Taufiqul Hakim, Md. Tanjil Islam Shovon: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

Md. Mirajul Islam, Md. Samiul Islam: Performed the experiments.

Md. Asadul Islam: Conceived and designed the experiments.

Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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