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M. A. Sorokina[1], **I. V. Korshukov**[1]*, **A.** E**. Tursynbek**[1]

IN SILICO ANALYSIS OF RARE PATHOGENIC MISSENSE VARIANTS IN THE SLC6A2 GENE

[1]Institute of Life Sciences, Department of Informatics and Biostatistics, Karaganda Medical University NC JSC (100008, Republic of Kazakhstan, Karaganda c., 40 Gogolya str., 40; e-mail: info@qmu.kz)

***Ilya Vasilyevich Korshukov** – Institute of Life Sciences, Department of Informatics and Biostatistics Karaganda Medical University NC JSC (100008, Republic of Kazakhstan, Karaganda c., 40 Gogolya str., 40; e-mail: Korshukov@qmu.kz

Background. The sodium-dependent noradrenaline transporter (NET), encoded by the SLC6A2 gene, plays a key role in the regulation of noradrenergic transmission and is associated with orthostatic intolerance, attention-deficit/hyperactivity disorder (ADHD), depression, and autonomic dysfunctions. The emergence of cryo-EM structures of human NET in complex with noradrenaline and antidepressants, along with new machine-learning algorithms, has enabled systematic *in silico* screening of known missense variants of the gene and the identification of previously uncharacterized pathogenic substitutions that may explain cases of idiopathic orthostatic intolerance, treatment-resistant depression, paradoxical responses to therapy, or hereditary autonomic disorders.

Aim of the study was to analyze missense variants of the SLC6A2 gene registered in the NCBI database (assembly GRCh38.p14), to identify variants with the highest predicted pathogenicity scores, and to perform their structural-functional characterization using modern bioinformatics tools.

Materials and methods. From 725 missense single-nucleotide variants (SNVs) of unknown clinical significance in the SLC6A2 gene (GRCh38), biallelic variants with the highest pathogenicity scores (AlphaMissense ≥0.99 and LIST-S2 ≥0.99) were selected. Two rare variants (rs1397308523 (Pro108Thr) and rs759975667 (p.Gln314Arg) underwent comprehensive *in silico* analysis.

Results and discussion. Both substituted residues are highly conserved (ConSurf 9/9). Predictions of topology (DeepTMHMM) and domain organization (PROSITE) revealed no differences from the wild type. MutPred2 assigned overall pathogenicity scores of 0.87 and 0.91, respectively. I-Mutant 2.0 and MUpro predicted decreased protein stability (ΔΔG –1.11 and –0.94 kcal/mol).

Conclusions. The variants rs1397308523 and rs759975667, which have the highest predicted pathogenicity among missense substitutions in SLC6A2, represent priority targets for functional studies and screening in clinical cohorts.

Key words: SLC6A2; noradrenaline transporter; rare missense variants; in silico; pathogenicity; protein stability; ConSurf; MutPred2

INTRODUCTION

The sodium-dependent norepinephrine transporter (NET, also known as NAT or SLC6A2) is a transmembrane protein of the solute carrier 6 (SLC6) family that mediates Na^+/Cl^--dependent reuptake of norepinephrine (and partially dopamine) from the synaptic cleft into presynaptic nerve terminals of sympathetic neurons and CNS neurons. NET represents the only physiological mechanism for rapid inactivation of norepinephrine in most noradrenergic synapses, making it a key regulator of the sympathetic nervous system, blood pressure, heart rate, attention, mood, and stress responses [10, 16, 18, 26, 32].

The SLC6A2 gene is located on chromosome 16q12.2, consists of 15 exons, and encodes a protein of 617 amino acids (≈69 kDa) with the characteristic SLC6 topology comprising 12 transmembrane domains (TMDs), large extracellular and intracellular loops, and N- and C-terminal tails. NET belongs to the subfamily of sodium- and chloride-dependent neurotransmitter symporters, showing high homology with DAT (SLC6A3) and SERT (SLC6A4). However, the SLC6A2 gene has a unique feature – the presence of an additional exon encoding the C-terminal fragment – which distinguishes it from most members of the family and determines specific interactions with PDZ-domain-containing proteins (e.g., PICK1) and the regulation of intracellular trafficking [18, 27].

NET function is tightly regulated at multiple levels: post-translational modifications (dynamic

palmitoylation of Cys44 and other residues, phosphorylation, glycosylation) [4]; protein-protein interactions (PICK1, PP2A, syntaxin 1A); lipid microenvironment and dimerization through a cholesterol-dependent interface [26, 32]; pharmacological modulation (selective inhibitors - reboxetine, atomoxetine; tricyclic antidepressants, cocaine).

Dysfunction of NET underlies a wide spectrum of clinical conditions: monogenic hyperadrenergic syndromes, such as orthostatic intolerance and postural orthostatic tachycardia syndrome – a classic example being the A457P mutation (TMD9) [10, 23]; psychiatric disorders: attention deficit hyperactivity disorder (ADHD), depression, bipolar disorder, post-traumatic stress disorder (PTSD), panic attacks [12, 28]; cardiovascular diseases: essential hypertension, arrhythmias, sudden cardiac death; neurodegenerative processes (Parkinson's disease – loss of NET marker in the locus coeruleus).

Furthermore, genetic variability in SLC6A2 determines individual responses to widely used drugs (norepinephrine reuptake inhibitors (SNRIs, atomoxetine) and psychostimulants) making this gene an important target for personalized medicine in psychiatry and cardiology.

The emergence of cryo-EM structures of human NET in complex with norepinephrine and antidepressants (2024 – 2025, resolution 2.5-3.2 Å) [26, 32], along with new machine learning algorithms (AlphaMissense, trained on >400 million missense variants; the ensemble predictor LIST-S2, 2024-2025), has enabled systematic *in silico* screening of all known missense variants in the gene and the identification of previously uncharacterized pathogenic substitutions that may explain cases of idiopathic orthostatic intolerance, treatment-resistant depression, paradoxical responses to therapy, or hereditary autonomic disorders.

The aim of the present study was to analyze missense variants of the SLC6A2 gene registered in the NCBI database (assembly GRCh38.p14), to identify variants with the highest predicted pathogenicity scores, and to perform their structural-functional characterization using modern bioinformatics tools.

MATERIALS AND METHODS

Data were obtained from the NCBI Variation Viewer (assembly GRCh38.p14). Missense single-nucleotide variants of unknown clinical significance were selected. The Variant Effect Predictor (VEP) with the dbNSFP plugin was additionally used to obtain pathogenicity predictions from AlphaMissense and LIST-S2. SNVs were selected based on a consensus score from these methods (pathogenicity score ≥0.99).

Population frequencies, specifically global and population-specific MAF, were retrieved from gnomAD v4 and ALFA via NCBI.

Conservation analysis was performed using the ConSurf Server [2] (150 homologs, CSI-BLAST + MAFFT) with the structure 8WTV.

Prediction of membrane topology and domain structure was carried out using DeepTMHMM 1.0 [11] and ScanProsite (PROSITE) [24].

For pathogenicity prediction and mechanistic insights, MutPred2 (web version) [17], I-Mutant 2.0 (structure-based mode, pH 7.5, PDB 8Y2D) [6], and MUpro were employed [8].

RESULTS

Search and primary filtering of missense variants in the SLC6A2 gene

A search for missense variants of the SLC6A2 gene was conducted in the NCBI Variation Viewer database (assembly GRCh38.p14). After applying the filters «single nucleotide variant», «missense variant», and «clinical significance: uncertain significance / not provided», 725 missense variants were obtained. For further analysis, 152 biallelic variants were selected.

Prediction of pathogenicity and final selection

For all 152 biallelic missense variants, pathogenicity predictions were obtained using the Variant Effect Predictor (VEP) with the dbNSFP plugin, employing two state-of-the-art algorithms (AlphaMissense and LIST-S2). Threshold values were set for the two most accurate next-generation meta-predictors: AlphaMissense_score ≥0.99 and LIST-S2_score ≥0.99. Only two variants simultaneously met these criteria: rs1397308523 (chr16:55669612 C>A, c.322 C>A, p.Pro108Thr); rs759975667 (chr16:55694032 A>G, c.941 A>G, p.Gln314Arg).

Both variants are recorded as single findings in the gnomAD v4 and ALFA databases. The global minor allele frequency is $<10^{-6}$ and they are absent in major populations.

Evolutionary conservation

The evolutionary conservation of the substituted residues was assessed using the ConSurf web server. Homologs were searched using CSI-BLAST (E-value 0.0001, 3 iterations), multiple sequence alignment was performed with the MAFFT algorithm, and 150 unique homologous sequences were selected. The cryo-EM structure of human NET in complex with norepinephrine (PDB ID: 8WTV, chain A) [32] was used as a template for mapping conservation onto the three-dimensional structure. The resulting conservation scores were as follows: Pro108 – 9/9 (functionally/structurally important, buried) and Gln314 – 9/9.

Influence on membrane topology and domain organization

Topology prediction using DeepTMHMM 1.0 revealed no differences between the wild-type and mutant sequences: in all cases, the classical SLC6 topology is preserved – 12 transmembrane domains (TMD1–TMD12), large extracellular and intracellular loops, with correctly oriented N- and C-terminal

Table 1 – Predicted molecular mechanisms based on MutPred2

Variant	Molecular mechanism (in descending order of probability)	Pr	p-value
p.Pro108Thr	Altered Ordered interface	0.26	0.02
	Altered Transmembrane protein	0.18	7.4e-03
	Loss of catalytic site at E113	0.17	0.02
	Altered Metal binding	0.14	0.04
p.Gln314Arg	Altered Transmembrane protein	0.29	2.1e-04
	Altered Ordered interface	0.25	0.03
	Altered Metal binding	0.15	0.04
	Loss of Pyrrolidone carboxylic acid at Q314	0.04	0.05

tails. Scanning in PROSITE confirmed complete preservation of the functional profile «sodium: neurotransmitter symporter» (PS50268, E-value $<10^{-40}$) in the sequences studied.

Overall pathogenicity and molecular mechanisms according to MutPred2 are presented in Table 1.

MutPred2 assigned high overall pathogenicity scores: p.Pro108Thr – 0.87 (very likely pathogenic) and p.Gln314Arg – 0.91 (very likely pathogenic).

According to the data presented in Table 1, both variants disrupt the structure and stability of NET transmembrane domains and alter metal-binding properties and ordered interfaces. For p.Pro108Thr, an additional loss of catalytic function near position Glu113 is predicted, while for p.Gln314Arg, disruption of glutamine modification (potentially related to post-translational processes) is predicted.

Prediction of protein stability change

Two independent algorithms (I-Mutant 2.0 and MUpro) predicted decreased stability of the mutant forms (Table 2).

As demonstrated by high-resolution cryo-EM structures of the human norepinephrine transporter [26, 32], the proline at position 108 is part of the structural scaffold between the first and second transmembrane domains and directly adjoins the extracellular «gate» that controls access to the central S1 binding site. The glutamine at position 314 is located in the extracellular loop EL4 and participates in forming the Na2 ion-binding site, which plays a key role in the protein's conformational transitions. Disruption of these critical elements is fully consistent with the predicted pathogenicity of the identified missense substitutions.

Thus, a comprehensive multilevel *in silico* analysis enabled the identification of two rare SLC6A2 missense variants (rs1397308523 and rs759975667) as having high pathogenicity scores and a predicted mechanism of action involving protein destabilization and disruption of NET conformational dynamics.

DISCUSSION

The two identified rare missense variants, rs1397308523 (p.Pro108Thr) and rs759975667 (p.Gln314Arg), are SLC6A2 variants that simultaneously meet the strict modern *in silico* criteria for likely pathogenicity (AlphaMissense ≥0.99 and LIST-S2 ≥0.99). This makes them priority candidates for further investigation.

Comparison with known pathogenic variants

The well-established mutation A457P (TMD9) is the only proven cause of monogenic orthostatic intolerance; it leads to near-complete loss of NET function due to misfolding, endoplasmic reticulum retention, and a dominant-negative effect on the wild-type protein [10, 23]. In contrast, the variants studied here preserve the overall topology of 12 transmembrane domains and the core functional symporter profile (PROSITE PS50268), while protein stability is only moderately reduced ($\Delta\Delta G \approx -1$ kcal/mol). This suggests partial loss of function: a phenotype that may not manifest as classic severe orthostatic intolerance but rather as milder or atypical forms – mild orthostatic intolerance, POTS, partial resistance to NET inhibitors, episodic tachycardia, anxiety disorders, or impaired thermoregulation.

Structural-functional consequences of the specific substitutions

As in other SLC6 family transporters, the proline at position 108 is the only amino acid that creates a rigid kink in the transmembrane domain, which is

Table 2 – Characteristics of the stability of mutant forms

Variant	I-Mutant 2.0 (ΔΔG, kcal/mol)	MUpro (ΔΔG, kcal/mol)	Prediction
p.Pro108Thr	–1.11	–0.94	Decreased stability
p.Gln314Arg	–0.94	–0.97	Decreased stability

Note: ΔΔG values < –0.5 kcal/mol are considered clinically significant for membrane proteins

essential for conformational alternation [26, 28, 32]. Substitution with threonine (a polar, flexible residue bearing a hydroxyl group) may disrupt local geometry, which is critical for conformational switching. The introduction of a hydroxyl group could create a new site for O-glycosylation or phosphorylation, thereby altering intracellular trafficking of NET – similar to what has been shown for glycosylation of DAT in the SLC6 family [12].

The introduction of a bulky, positively charged guanidino group from arginine into a region normally occupied by a neutral glutamine can disrupt electrostatic interactions with neighboring residues or with the lipid bilayer. Additionally, arginine may serve as a new site for ubiquitination or SUMOylation, thereby accelerating protein degradation - a mechanism already described for pathogenic missense variants in DAT (SLC6A3) and SERT (SLC6A4) [15, 22].

Clinical-genetic significance and prospects

In cohorts of patients with POTS, the frequency of pathogenic SLC6A2 variants is estimated at 2-8% [3, 10, 23, 33]. The variants studied here are potential candidates for targeted sequencing in such cohorts.

Partial NET dysfunction caused by rare variants may be one of the reasons for an inadequate therapeutic response to serotonin-norepinephrine reuptake inhibitors (SNRIs; venlafaxine and duloxetine) in patients with depression and generalized anxiety disorder [28, 19, 25, 31]. When transporter activity is reduced, baseline extracellular norepinephrine levels are already elevated, which diminishes the additional effect of NET blockade and leads to a clinically significant weakening of the antidepressant action.

Similarly, in some patients with ADHD, administration of the selective NET inhibitor atomoxetine paradoxically exacerbates anxiety, irritability, or even triggers panic attacks instead of the expected improvement [5, 12, 14, 20]. This phenomenon has repeatedly been associated with genetically determined reduction in NET density or function: when norepinephrine reuptake is already impaired, additional transporter blockade results in excessive accumulation of the neurotransmitter in the synaptic cleft and hyperactivation of α_1- and β-adrenoceptors, particularly in the prefrontal cortex and amygdala [12, 20].

Thus, the variants identified in this study (rs1397308523 (p.Pro108Thr) and rs759975667 (p.Gln314Arg) are promising candidates for pharmacogenetic testing when selecting therapy with venlafaxine, duloxetine, and atomoxetine, and may also explain cases of treatment-resistant depression and paradoxical reactions to standard ADHD treatment regimens.

It has previously been shown that gain-of-function variants in the SLC6A2 gene are associated with endurance and high performance in professional athletes [9]. In contrast, the loss-of-function variants identified in this study may predispose individuals to rapid fatigue during prolonged physical exertion, as well as increased fatigue and difficulty recovering under chronic stress.

Limitations of the study and future directions

Despite the high predictive power of the algorithms used (AlphaMissense trained on >400 million missense variants [7]; LIST-S2 – an ensemble meta-predictor from 2024 – 2025 [13]), definitive classification as «pathogenic» requires functional validation according to ACMG/AMP criteria [21].

Future experiments could include:

- heterologous expression of mutant NET constructs in HEK293 and PC12 cell lines with measurement of surface expression (biotinylation), Vmax, and Km of [^{3}H]-norepinephrine uptake [10, 28];
- assessment of mutant protein thermostability using the CPM assay [1];
- targeted CRISPR/Cas9 editing in induced pluripotent stem cells (iPSCs) followed by differentiation into sympathetic neurons and subsequent phenotyping [29, 30].

Thus, the *in silico* screening of SLC6A2 missense variants identified two rare variants that are pathogenic. Their phenotypic manifestations fall within the spectrum of partial NET dysfunction and may account for idiopathic cases of orthostatic, psychiatric, and autonomic disorders. The obtained data justify the inclusion of full SLC6A2 sequencing in diagnostic panels for POTS, idiopathic orthostatic intolerance, and treatment-resistant affective disorders, while also paving the way for personalized therapy with NET inhibitors and modulators.

Given their predicted destabilizing effect on NET structure, the studied variants may in the future serve as candidates for:

- in vitro functional studies;
- targeted screening in patient cohorts with idiopathic POTS, treatment-resistant depression, and ADHD;
- pharmacogenetic testing of response to NET inhibitors (atomoxetine and others).

Author contributions:

M. A. Sorokina, I. V. Korshukov – study concept and design.

I. V. Korshukov, A. Tursynbek – data collection and processing.

M. A. Sorokina, I. V. Korshukov, A. Tursynbek – data analysis.

M. A. Sorokina, A. Tursynbek – writing the manuscript.

M. A. Sorokina, I. V. Korshukov – editing.

Conflict of interest:

The authors declare no conflict of interest.

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М. А. Сорокина[1], И. В. Коршуков[1], А. Е. Турсынбек[1]*

IN SILICO АНАЛИЗ РЕДКИХ ПАТОГЕННЫХ МИССЕНС-ВАРИАНТОВ ГЕНА SLC6A2

[1]Институт наук о жизни, кафедра информатики и биостатистики НАО «Карагандинский медицинский университет» (100008, Республика Казахстан, г. Караганда, ул. Гоголя 40; e-mail: info@qmu.kz)

***Илья Васильевич Коршуков** – Институт наук о жизни, кафедра информатики и биостатистики НАО «Карагандинский медицинский университет»; 100008, Республика Казахстан, г. Караганда, ул. Гоголя 40; e-mail: Korshukov@qmu.kz

Актуальность. Натрий-зависимый транспортер норадреналина (NET), кодируемый геном SLC6A2, играет ключевую роль в регуляции норадренергической передачи и ассоциирован с ортостатической непереносимостью, синдромом дефицита внимания и гиперактивности (СДВГ), депрессией и автономными нарушениями. Появление крио-ЭМ структур NET человека в комплексе с норадреналином и антидепрессантами, а также новых алгоритмов машинного обучения открыло возможность систематического *in silico* скрининга известных миссенс-вариантов гена и выявления ранее не охарактеризованных патогенных замен, которые могут объяснять случаи идиопатической ортостатической непереносимости, резистентной депрессии, парадоксального ответа на терапию или наследуемых автономных расстройств.

Цель. Анализ миссенс-вариантов гена SLC6A2, зарегистрированных в базе NCBI (сборка GRCh38.p14), выделение вариант с наивысшими предсказаниями патогенности и их структурно-функциональных характеристика с использованием современных биоинформатических инструментов.

Материалы и методы. Из 725 миссенс однунклеотидных вариантов (ОНВ) с неизвестной клинической значимостью для гена SLC6A2 (GRCh38) были отобраны биаллельные варианты с наибольшими оценками патогенности (AlphaMissense ≥0,99 и LIST-S2 ≥0,99). Два редких варианта (rs1397308523 (Pro108Thr) и rs759975667 (p.Gln314Arg) были подвергнуты комплексному *in silico* анализу.

Результаты и обсуждение. Оба замещаемых остатка высококонсервативны (ConSurf 9/9). Прогноз топологии (DeepTMHMM) и доменной организации (PROSITE) не выявил различий с диким типом. MutPred2 оценил общую патогенность в 0,87 и 0,91 соответственно. I-Mutant 2.0 и MUpro предсказали снижение стабильности белка (ΔΔG –1,11 и –0,94 ккал/моль).

Выводы. Варианты rs1397308523 и rs759975667, имеющие наибольшую прогнозируемую патогенность, миссенс-замены в SLC6A2 представляют приоритетные мишени для функциональных исследований и поиска в клинических когортах.

Ключевые слова: SLC6A2; транспортер норадреналина; редкие миссенс-варианты; in silico; патогенность; стабильность белка; ConSurf; MutPred2

М. А. Сорокина[1], И. В. Коршуков[1], А. Е. Турсынбек[1]*

SLC6A2 ГЕНІНІҢ СИРЕК ПАТОГЕНДІК МИССЕНС-ВАРИАНТТАРЫНЫҢ IN SILICO ТАЛДАУЫ

[1]«Қарағанды медицина университеті» КеАҚ өмір туралы ғылымдар институты, информатика және биостатистика кафедрасы (100008, Қазақстан Республикасы, Қарағанды қ., Гоголь көш., 40; e-mail: info@qmu.kz)

***Илья Васильевич Коршуков** – «Қарағанды медицина университеті» КеАҚ өмір туралы ғылымдар институты, информатика және биостатистика кафедрасы; 100008, Қазақстан Республикасы, Қарағанды қ., Гоголь көш., 40; e-mail: Korshukov@qmu.kz

Өзектілік. SLC6A2 генімен кодталатын, натрийге тәуелді норадреналин тасымалдаушысы (NET), норадренергикалық берілімді реттеудегі негізгі рөл атқарады және ол ортостатикалық төзімсіздік, назар тапшылығы және гиперактивтілік синдромы (НТГС), депрессия және автономдық бұзылыстармен байланысты. Адамның NET норадреналин және антидепрессанттар кешеніндегі крио-ЭМ құрылымдарының пайда болуы, жаңа машиналық оқыту алгоритмдері геннің белгілі миссенс-варианттарына in silico жүйелі скринингтеу мүмкіндігін ашты, сондай-ақ бұрын сипатталмаған патогендік ауыстыруларды анықтауға мүмкіндік беріп, идиопатикалық ортостатикалық төзімсіздік, терапияға төзімді депрессия, терапияға парадоксалды жауап немесе мұрагерлік автономдық бұзылыстардың жағдайларын түсіндіре алады.

Зерттеудің мақсаты. SLC6A2 генінің NCBI дерекқорында (GRCh38.p14 жинағы) тіркелген миссенс-варианттарын талдау, патогенділік бойынша ең жоғары болжамдарға ие варианттарды бөліп көрсету және оларды заманауи биоинформатикалық құралдар арқылы құрылымдық-функционалдық сипаттау.

Материалдар және әдістер. SLC6A2 гені (GRCh38) үшін клиникалық маңызы белгісіз 725 миссенс бірнуклеотидті варианттарының (БНВ) ішінен патогенділіктің ең жоғары бағалары бар (AlphaMissense ≥0,99 және LIST-S2 ≥0,99) биаллельді варианттар таңдалды. Екі сирек вариант – rs1397308523 (Pro108Thr) және rs759975667 (p.Gln314Arg) – кешенді *in silico* талдауына ұшыратылды.

Нәтижелер және талқылау. Екі ауыстырылатын қалдық та жоғары консервативті (ConSurf 9/9). Топологияның болжамы (DeepTMHMM) және домендік ұйымдастырудың болжамы (PROSITE) жабайы типтен ешқандай айырмашылықты көрсетпеді. MutPred2 жалпы патогенділікті тиісінше 0,87 және 0,91 деп бағалады. I-Mutant 2.0 және MUpro ақуыз тұрақтылығының төмендеуін болжады (ΔΔG –1,11 және –0,94 ккал/моль).

Қорытындылар. Ең жоғары болжамдалған патогенділікке ие rs1397308523 және rs759975667 миссенс-ауыстырулар, SLC6A2-дегі функционалдық зерттеулер мен клиникалық когорттардағы ізденім үшін басым нұсқа болып табылады.

Кілт сөздер: SLC6A2; норадреналин тасымалдаушысы; сирек миссенс-варианттары; in silico; патогенділік; ақуыз тұрақтылығы; ConSurf; MutPred2