NTNG2 Mutation: A candidate gene for a new brain-skin disorder with early neuropsychiatric manifestation? An analysis based on 3000 patients

NTNG2 Mutasyonu: Erken nöropsikiyatrik manifestasyonlu yeni bir beyin-cilt hastalığı için aday bir gen mi? 3000 hasta üzerinden bir analiz.

Burak Yuluğ¹, Akif Ayaz²*

1.Department of Neurology, Alaaddin Keykubat University Faculty of Medicine, Alanya, Turkey
2.Department of Medical Genetics, Istanbul Medipol University Faculty of Medicine, Istanbul, Turkey

ABSTRACT

Aim: In this study, the relationship between genetic analysis and exome sequencing and clinical and neuroimaging findings of four patients from the same family was investigated by analyzing a clinical and genetic (WES) database containing more than 3000 patients.
Methods: We analyzed the WES data of approximately 3000 patients performed in our center in terms of NTNG2 biallelic mutations. In addition, MR imaging findings were investigated.
Results: We found four patients with the same mutation in the NTNG2 gene, presenting with similar clinical and neuroimaging findings. As a result of filtering, the c242G>A variant was determined in the NTNG2 gene. In addition, mild to severe brain parenchymal volume loss and frontal and temporal lobe atrophy were seen in cases 1, 2, and 4 on axial T2-weighted MRI.
Conclusion: The current study has similar phenotypic and genotypic features and is a very rare report showing NTNG2 mutation in this context. Existing clinical data are important in choosing NTNG2 gene-related neuropsychiatric disorders as a future treatment target.

Keywords: Netrin-g2; Synapse formation; Phenotype; WES analysis; Schizophrenia; Neuropsychiatric Disease

ÖZ

Amaç: Bu çalışmada 3000'den fazla hastayı içeren klinik ve genetik (WES) veri tabanını analiz ederek aynı aileden dört hastanın genetik analizi ve ekzom dizilimi ile klinik ve nörogörüntüleme bulguları arasındaki ilişki araştırılmıştır.
Yöntem: Merkezimizde 3000 hastanın WES verileri NTNG2 bialelik mutasyonları açısından incelendi. Ayrıca MR görüntüleme bulguları araştırıldı.
Bulgular: NTNG2 geninde aynı mutasyona sahip, benzer klinik ve nörogörüntüleme bulguları ile başvuran dört hasta bulundu. Filtreleme sonucunda NTNG2 geninde c.242G>A varyantı belirlendi. Ayrıca aksiyel T2 ağırlıklı MRG'de vaka 1, 2 ve 4'te hafif ila şiddetli beyin parankimal hacim kaybı ve frontal ve temporal lob atrofisi görüldü.
Sonuç: Mevcut çalışma benzer fenotipik ve genotipik özelliklere sahip olup bu bağlamda NTNG2 mutasyonunu gösteren çok nadir bir rapordur. Mevcut klinik data NTNG2 geniyle ilişkili nöropsikiyatrik bozukluklarda gelecekteki bir tedavi hedefi olarak seçilmesinde öneme sahiptir.

Anahtar Kelimeler: Netrin-g2, Sinaps oluşumu; Fenotip; WES analizi; Şizofreni; Nöropsikiyatrik hastalık

*Corresponding Author: Akif AYAZ, Department of Medical Genetics, Istanbul Medipol University Faculty of Medicine, Istanbul, Turkey. +902164448544, aayaz@medipol.edu.tr

ORCID: 0000-0001-6930-7148

INTRODUCTION

Netrin-g2 (Ntng2) also called laminet-2, a vertebrate-specific axon guidance molecule that belongs to the Netrin-G subfamily, binds to the plasma membrane through a glycosyl phosphatidylinositol (GPI) anchor [1,2,3]. The netrin-G1 (*Ntng1*) and *Ntng2* genes are expressed in the mouse brain in a complementary manner in the dorsal thalamus, olfactory bulb and the cerebral cortex, respectively [1,3]. Several studies have shown that NTNG2 can regulate synapse formation and promote neurite outgrowth [4, 5]. Also, its ligand, NetrinG2 ligand (NGL-2), supports presynaptic differentiation in cultured neurons probably via netrin-G2 [5]. Various expression studies of NTNG2 have reported its possible association with many phenotypes [1,7]. Recently, Zhang et al. reported that abnormal expression of Netrin-g2 and its receptor is associated with impaired memory, learning, and abnormal acoustic startle response in transgenic mice [6]. In their interesting paper, the authors have stated that Netrin-G2 and netrin-G2 ligand are both required for normal auditory responsiveness. On the other hand, abnormal expression levels, have also been reported to be associated with schizophrenia and bipolar disorder in human patients [7]. Also, single nucleotide polymorphisms (SNPs) in the genes for *NTNG1* and *NTNG2* have been reported to be associated with schizophrenia [8,9]. It has been reported that possible candidate genes for schizophrenia might have a role in synapse formation, functioning and plasticity [10,11]. Despite these promising studies suggesting the role of the *NTNG2* gene in the pathophysiology of schizophrenia, more detailed studies are needed to understand the specific symptoms of schizophrenia and bipolar disorder (BPD) (i.e., cognitive symptoms). Based on analysis of more than 3000 patients, in the current paper, we aimed to present the clinical and neuroimaging findings and results of whole exome sequencing of four patients belonging to the same family and present with severe psychomotor retardation. With this report, we hope to provide important informative data for the clinical findings caused by the *NTNG2*gene which could have therapeutic implications in the near future.
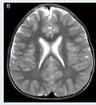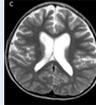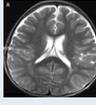
Clinical Presentation

A five-year-old boy (Case-1) was admitted to our department due to severe motor retardation and mutism. The patient has never walked and was not able to sit without support at five-year-old. Since he had respiratory distress immediately after delivery, he remained in the incubator with appropriate respiratory support for 2 days. In his detailed history, his parents have noticed that he suffered from hypotonia first time when he was four-month-old. At his current physical examination bi-temporal narrowing, bilateral strabismus, triangular face, skin hyperelasticity, hirsutism on arms and back, muscular hypotonia and hypoactive deep tendon reflex were present. He had also prominent torticollis, right SCM contraction without any contracture. Dysmorphic facial findings were mild and there was no abnormality in hand, foot and chest examination (Table 1). Cranial Magnetic Resonance Imaging (MRI) showed that the depth of the cerebral sulcus is slightly increased in the frontotemporal region indicating to an underlying mild cerebral atrophy (Figure 2). In his detailed family history, the parents have reported that they have first cousin relationship and the patient has also a sister who have similar clinical findings (Table 1). The second case was his sister (Case-II), who was eight years old and has never been able to speak and walk. Her clinical findings which were associated with significant general atrophy in the frontotemporal region (Figure 2) have been summarized in Table 1. Interestingly, there were similar findings in both cousins, fifteen-month-old (Case-III) male and eleven-year-old male (Case-IV), and there was also a first cousin relationship between their parents. Eleven year-old male presented with muscle atrophy and contracture and he has never been able to walk and speak. He had also bilateral undescended testis story, hirsutism, oculomotor apraxia, hypochromic microcytic anemia, patent ductus arteriosus and patent foramen ovale on echocardiography. In the cranial MRI taken at the age of three years, a slight increase in the CSF distances was observed in the anterior temporal lobe (Figure 2) and the frontal lobe. Accordingly, his cranial MRI revealed slight frontotemporal atrophy (Figure 2). Fifteen-month-old male patient showed mild facial dysmorphic findings such as the prominent forehead, synophrys and bilateral infraorbital creases, skin hyperelasticity

and prominent axial hypotonia. In his actual examination, the patient was not able to sit and to speak. Myotonic discharges have been observed on electromyography evaluation. Unfortunately, the MRI data was not available for this patient.

Shortly, after we have obtained the signed release from the patients, here we present four patients with *Ntng2* mutation who presented with similar clinical findings as well as a progressive cerebral atrophy pattern (Table 1 and Figure 2).

Table 1. Clinical, radiological and genetic findings of patients

		Case I	Case II	Case III	Case IV
Age		5 yo	8 yo	15 mo	11 yo
Gender		M	F	M	M
Onset of clinical findings		4 mo	4-6 mo	3-4 mo	4-6 mo
Neurological findings	Hypotonia	+	+	+	+
	Unsupported seating	-	-	-	-
	Walking	-	-	-	-
	Strabismus	+	-	-	-
	Speech	-	-	-	-
	Contracture	-	-	-	+
	Oculomotor apraxia	Unknown	Unknown	Unknown	+
Non-neurological findings	Skin hyperelasticity	+	+	+	+
	Intestinal motility disorder	-	+	-	-
	Cryptorchidism	-		-	-
	Hirsutism	+	-	-	+
	Hypochromic microcytic anemia	-	-	-	+
Cranial MR findings		Cerebral Atrophy	Cerebral Atrophy	NA	Cerebral Atrophy
EMG		NA	NA	Myotonic discharge	Normal
Echo		NA	NA	NA	PDA
WES Results		Homozygous c.242G>A NTNG2	Homozygous c.242G>A NTNG2	Homozygous c.242G>A NTNG2	Homozygous c.242G>A NTNG2

NA: Not available, PDA: Patent Ductus Arteriosus

Clinical, radiological and genetic findings of patients at different age groups. Please see common neurological and non-neurological findings in the table highlighted in red.

MATERIAL AND METHODS

We analyzed the WES data of approximately 3000 patients performed in our center in terms of *NTNG2* biallelic mutations. In this context, peripheral blood samples were collected at the Istanbul Medipol University and genomic DNA was isolated using standard protocols. Whole-exome sequencing was performed with the Illumina Nextera and with Illumina Nextseq 500 platform. Alignment to the reference genomes (hg19 for human) was performed using Burrows-Wheeler Aligner (BWA). We applied the following additional filters; the minimum read depth:10, the minimum base quality:20, the minimum of alternative allele frequency: %20. The identified variants were functionally annotated using ANNOVAR. We excluded from further analysis variants in non-coding regions, synonymous variants and variants present in highly repetitive regions. Detected variant in *NTNG2* was confirmed by Sanger Squencing with ABI 3130xl (Figure 1C). Written consent form was obtained from the parents of patients.
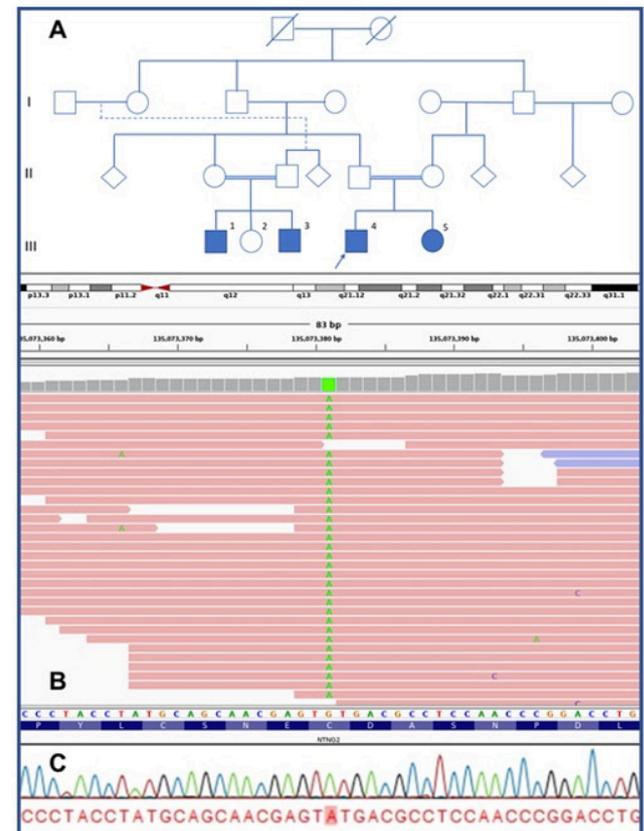

Figure 1. A. Affected cases in the family are shown with III-1, III-3, III-4 and III-5 in pedigree. B. IGV images of III-4 showing the homozygous G> A change C. Sanger sequencing of case III-4 supporting IGV image.
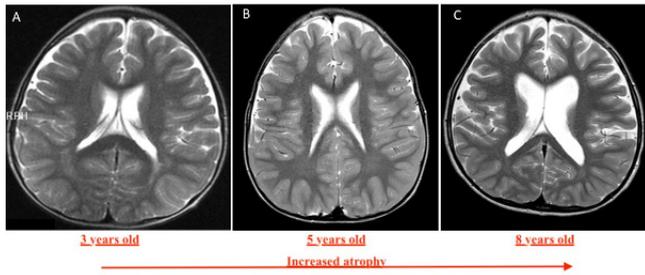
Figure 2. A. Cranial MR images of case III-1, at 3 years old, showing enlargement of CSF distances in the temporal lobe anterior. B. Cranial MRI images of case III-4 showing slightly increased in the frontotemporal region which could indicate to an underlying mildly cerebral atrophy (at 5 years old) C. Cranial MRI images of case III-5 showing significant general atrophy especially in frontotemporal region and dilated lateral ventricles (at 8 years old).

MRI: MR imaging was performed on 1.5-T MRI scanner (Magnetom Avanto, 18 channels, Siemens Medical Solutions, Erlangen, Germany) with a matrix head coil used as both transmitter and receiver. T1W, T2W, diffusion-weighted, and HEMO sequences were obtained in axial plane with 5 mm slice thickness and 30% interslice gap. For dedicated study, inversion recovery (IR) oblique coronal images (TE: 51, TR: 3500, FOV: 250 mm, slice thickness: 2 mm) and oblique coronal T2W images (TR: 4000, TE: 101, FOV: 230, slice thickness: 2 mm) covering the whole brain were acquired. The images were assessed for cortical atrophy, loss of defined morphologic structure of any specific region, increased T2W signal and decreased T1W signal. The diagnosis of the atrophy was made if there was evidence of signal abnormality of the specific region. Raters were blinded to the clinical information and each other's results.

RESULTS

In evaluating approx. 3000 patients WES data, we have found four patients from the same family who have the same mutation in *NTNG2* gene and present with similar clinical and neuroimaging findings: A 5-year-old male patient. There was no variant which might be enough to explain his clinical findings. Thus, we included other affected three family members to WES study and matched the homozygous common variants in all 4 patients. As a result of our filtering we have determined c.242G> A variant in *NTNG2* gene. We identified this variant as a heterozygote by sanger sequencing in the parents of patients and in a

healthy sibling. In addition, sanger confirmations of this variant were performed in patient DNAs. (Figure 1C)

MRI: Axial T2 weighted MRI showed mild to severe brain parenchymal volume loss and frontal and temporal lobe atrophy in case 1, 2 and 4. (Figure 2).

DISCUSSION

Studies evaluating available NTNG2 data have reported that the NTNG2 gene promotes presynaptic differentiation in neurons co-cultured with its ligand (NGL-2) [5]. These findings were in accordance with expression studies, revealing that NTNG2 and its receptor have been associated with impaired memory and learning in schizophrenia and bipolar disorder [6,7]. Wei G. et al. reported that KDM5C mutations regulate Netrin G2 and suppress neurite growth in Neuro2a cells [12] . In the light of these findings, it is not unreasonable to assume that the most likely mechanism to explain our patient's findings might be the impaired synaptogenesis which is one of the important components of brain development, especially when it comes to neurogenesis and migration. It is well-known that synaptogenesis involves the formation and elimination of synapses over time while the failure of this process may cause progressive clinical and neurobiological deterioration in various neuropsychiatric disorders. Here, we present clinical findings and the whole exome sequencing results of four patients who had similar clinical findings such as severe hypotonia, intellectual disability, motor retardation and skin hyperelasticity. Despite these similar presentations, the patients differed in terms of some clinical features which are summarized in Table 1. Radiologically, although there was no significant difference in any of the MRI atrophy parameters in the first years of life between the subjects, there was a trend towards an atrophy (i.e., increased extra-axial cerebrospinal fluid distance in frontotemporal region and decreased periventricular white matter volume in bi-frontoparietal region) in the following years (5-10-year-old) suggesting that there could be an underlying progressive neurodegenerative process. (Figure 2). Importantly, we detected homozygous c.242G>A (p.Cys81Tyr) variant on

NTNG2 gene which is not observed in WES data of approximately 3000 other patients that we have analyzed before. It should be noted that there was no problem in IGV images and the images were found to be normal in terms of reading depth and base quality (Figure 1B). Also, sanger squencing of this region was performed. This variant has not identified in gnomAD, exac and iranome databases. According to ACMG, it has PM1, PM2, PP5, PP2 and PP3 criteria and is classified as pathogenic. In silico analysis tools (MutationTaster, FATHMM, FATHMM-MKL, MetaSVM, MetaIR), all of them, were classified "damaging" or "disease causing". The variant of DANN score, located in highly conserved region, is 0.9973, and GERP score is 5. SIFT and Provean, which are two of mostly used functional in slico tools were classified the variant we detected as damaging (Table 2).

Clinical Databases	Clinvar	Pathogenic
	HGMD	Disease-causing mutations
	ACMG classification	Pathogenic (PM1, PM2, PP5, PP2, PP3)
Allel frequency	gnomAD exomes	--
	gnomAD genomes	--
	Iranome	--
	In house database	--
In silico predicton database	Mutation Taster	Disease causing
	FATHMM	Damaging
	FATHMM-MKL	Damaging
	MetaSVM	Damaging
	MetaIR	Damaging
Functional Database	SIFT	Damaging
	Provean	Damaging
DANN Score		0.9973
GERP Score		5

In addition, segregation of the variant in family members were compatible with autosomal recessive inheritance pattern. The strength of our present report is that although some studies on NTNG2 have been done at the molecular level, clinical findings are still very inconclusive. In light of current information, there are very few records on NTNG2 in HGMD. Taken together, with this current article, we aimed to clarify the clinical manifestations caused by *NTNG2* mutations in humans and evaluate if the *NTNG2* may be a good candidate gene for a new brain-skin disorder characterized with hypotonia, intellectual disability, cerebral atrophy and skin hyperelasticity. Except of one patient all MRI findings were available which were taken at different time points which can be considered as a major weakness in this study. Although we are aware that longitudinal MRI data gathered from each patient would provide a more reliable data, we were curious to see if the cross-sectional neuroimaging data differed between different age groups which could indicate to an underlying progressive neurodegenerative process. To this respect, instead of waiting for each patient's neuroimaging data, which is time-consuming, we preferred to compare the imaging data between patients in a time-dependent manner. Not surprisingly, we have revealed that these patients not only presented with similar atrophic regions in the MRI but also showed a progressive atrophy pattern which was associated with increased age. In order to accurately describe the progressive pathology, and overcome common problems of cross-sectional data we are aware of the fact that the emphasis of this study should be placed on longitudinal neuroimaging data (serial correlations, time-dependent interindividual variability). Despite this limitation our current findings provide first preliminary evidence for the neuropathophysiology of NTGN2 mutation. In addition, since the embryonic ectodermal germ layer contribute to central nervous system and skin organ systems, and many brain-skin disorders are strongly correlated with neurobehavioral impairment (i.e., Von Recklinghausen's disease) it is not surprising that our patients have been presented with common skin and central nervous system findings. Although there is still no evidence for the existence of NTGN2 expression in the skin tissue further studies should evaluate whether NTNG2 is involved during the embryonic ectodermal germ layer development. Given the neuromorphological evidence in our study, it is not unreasonable to assume that NTNG2 might play a critical role in schizophrenia which has been suggested to be a neurodevelopment disorder while frontal-lobe-related executive dysfunction and cognitive failure is usually noted during the course of schizophrenia [13]. Here it is interesting to notice that recent studies indicated that executive dysfunction was especially present in parents with a positive family history of schizophrenia while schizophrenia diagnosis in the family predicted

impaired performance on executive function in healthy relatives suggesting that hypofrontality may represent a genetic endophenotype for schizophrenia [14,15]. Accordingly, recent SNPs and haplotype studies have confirmed that there is a significant association between NTNG2 and schizophrenia [8]. Considering the anatomic and functional significance of the frontal cortex in the pathogenesis of schizophrenia [16], defects in any of the structures which are involved in intrinsic and extrinsic functional connectivity (i.e., DLPF circuit) might be related to the negative symptomology of schizophrenia such as the dysfunction of the attention and executive function [17]. In this context, the potential disturbance of NTN2 gene regulation at the transcriptional level might suggest a molecular contribution by netrin-G gene(s) to the disrupted higher-order brain functions in schizophrenia. It should be also noted that recent experimental studies have suggested that attention and cognitive deficits in schizophrenia might be related to impaired sensorimotor filtering [18, 19]. For instance, Nishumara et al have revealed that Ntng2 mutant mice showed altered NMDA receptor-mediated electrophysiologic responses in brain slices demonstrating that netrin family proteins are critical for NMDA receptor function, lending further support to altered NMDA neurotransmission hypothesis for schizophrenia [19]. Although we consider our findings to be significant confirmation of results by pooling data from multiple cohorts would be required. Moreover, further well-designed clinical neuroimaging studies (i.e Magnetic Resonance Spectroscopy) with larger number of human subjects in combined with relevant Induced Pluripotent Stem cell (IPS) culture studies would be logical steps to understand the pathophysiology of the underlying progressive cerebral atrophy. Although we are aware that the present clinical and neuroimaging data are not conclusive and need to be strengthened, we believe that its novelty (this is the first demonstration that NTNG2 phenotype is associated with common neuropsychiatric in all patients) deserves to be brought to the attention of the neuropsychiatry and neurogenetic community. The clinical presentation of *Ntng2* mutation reported here may even be considered as a future treatment target in *Ntng2* gene related neuropsychiatric disorders.

ORCID and Author contribution: **BY (0000-0002-9704-6173):** Concept and Design, Data collection, Analysis and Interpretation, Manuscript Writing, Critical Review. **AA (0000-0001-6930-7148):** Concept and Design, Literature search, Manuscript Writing, Critical Review.

REFERENCES

1. Nakashiba T, Nishimura S, Ikeda T, Itohara S. Complementary expression and neurite outgrowth activity of netrin-G subfamily members. Mech Dev. 2002;111(1-2):47-60. doi: 10.1016/s0925-4773(01)00600-1.

2. Nakashiba T, Ikeda T, Nishimura S, Tashiro K, Honjo T, Culotti JG, Itohara S. Netrin-G1: a novel glycosyl phosphatidylinositol-linked mammalian netrin that is functionally divergent from classical netrins. J Neurosci. 2000;20(17):6540-50. doi: 10.1523/JNEUROSCI.20-17-06540.2000.

3. Yin Y, Miner JH, Sanes JR. Laminets: laminin- and netrin-related genes expressed in distinct neuronal subsets. Mol Cell Neurosci. 2002;19(3):344-58. doi: 10.1006/mcne.2001.1089.

4. Lin JC, Ho WH, Gurney A, Rosenthal A. The netrin-G1 ligand NGL-1 promotes the outgrowth of thalamocortical axons. Nat Neurosci. 2003;6(12):1270-6. doi: 10.1038/nn1148.

5. Kim S, Burette A, Chung HS, Kwon SK, Woo J, Lee HW, Kim K, Kim H, Weinberg RJ, Kim E. NGL family PSD-95-interacting adhesion molecules regulate excitatory synapse formation. Nat Neurosci. 2006;9(10):1294-301. doi: 10.1038/nn1763.

6. Zhang W, Rajan I, Savelieva KV, Wang CY, Vogel P, Kelly M, Xu N, Hasson B, Jarman W, Lanthorn TH. Netrin-G2 and netrin-G2 ligand are both required for normal auditory responsiveness. Genes Brain Behav. 2008;7(4):385-92. doi: 10.1111/j.1601-183X.2007.00361.x.

7. Eastwood SL, Harrison PJ. Decreased mRNA expression of netrin-G1 and netrin-G2 in the temporal lobe in schizophrenia and bipolar disorder. Neuropsychopharmacology. 2008;33(4):933-45. doi: 10.1038/sj.npp.1301457

8. Aoki-Suzuki M, Yamada K, Meerabux J, Iwayama-Shigeno Y, Ohba H, Iwamoto K, Takao H, Toyota T, Suto Y, Nakatani N, Dean B, Nishimura S, Seki K, Kato T, Itohara S, Nishikawa T, Yoshikawa T. A family-based association study and gene expression analyses of netrin-G1 and -G2 genes in schizophrenia. Biol Psychiatry. 2005 Feb 15;57(4):382-93. doi: 10.1016/j.biopsych.2004.11.022.

9. Fukasawa M, Aoki M, Yamada K, Iwayama-Shigeno Y, Takao H, Meerabux J, Toyota T, Nishikawa T, Yoshikawa T. Case-control association study of human netrin G1 gene in Japanese schizophrenia. J Med Dent Sci. 2004 Jun;51(2):121-8. PMID: 15508520.

10. Harrison PJ, Owen MJ. Genes for schizophrenia? Recent findings and their pathophysiological implications. Lancet. 2003 Feb 1;361(9355):417-9. doi: 10.1016/S0140-6736(03)12379-3.

11. Harrison PJ, Weinberger DR. Schizophrenia genes, gene expression, and neuropathology: on the matter of their convergence. Mol Psychiatry. 2005;10(1):40-68; image 5. doi: 10.1038/sj.mp.4001558.

12. Wei G, Deng X, Agarwal S, Iwase S, Disteche C, Xu J. Patient Mutations of the Intellectual Disability Gene KDM5C Downregulate Netrin G2 and Suppress Neurite Growth in Neuro2a Cells. J Mol Neurosci. 2016;60(1):33-45. doi: 10.1007/s12031-016-0770-3.

13. Holmén A, Juuhl-Langseth M, Thormodsen R, Ueland T, Agartz I, Sundet K, Andreassen OA, Rund BR, Melle I. Executive function in early- and adult onset schizophrenia. Schizophr Res. 2012;142(1-3):177-82. doi: 10.1016/j.schres.2012.10.006.

14. Wong AH, Van Tol HH. Schizophrenia: from phenomenology to neurobiology. Neurosci Biobehav Rev. 2003;27(3):269-306. doi: 10.1016/s0149-7634(03)00035-6.

15. Aydın E, Cansu Ülgen M, Tabo A, Devrim Balaban Ö, Yeşilyurt S, Yumrukçal H. Executive function and genetic loading in nonpsychotic relatives of schizophrenia patients. Psychiatry Res. 2017;248:105-110. doi: 10.1016/j.psychres.2016.12.027.

16. Erol A, Bayram S, Kosger F, Mete L. Executive functions in patients with familial versus sporadic schizophrenia and their parents. Neuropsychobiology. 2012;66(2):93-9. doi: 10.1159/000337738.

17. Bunney WE, Bunney BG. Evidence for a compromised dorsolateral prefrontal cortical parallel circuit in schizophrenia. Brain Res Brain Res Rev. 2000;31(2-3):138-46. doi: 10.1016/s0165-0173(99)00031-4.

18. DL, Geyer MA. Sensorimotor gating and schizophrenia. Human and animal model studies. Arch Gen Psychiatry. 1990;47(2):181-8. doi: 10.1001/archpsyc.1990.01810140081011.

19. Woo J, Kwon SK, Kim E. The NGL family of leucine-rich repeat-containing synaptic adhesion molecules. Mol Cell Neurosci. 2009 Sep;42(1):1-10. doi: 10.1016/j.mcn.2009.05.008.