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HUMAN GENETICS

Replicative Study of Susceptibility to Childhood-Onset Schizophrenia in Kazakhs

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Abstract—This paper reports the results of replicative analysis of associations of 15 SNPs in a region of 14 genes previously identified in genome-wide association studies (GWAS) with early-onset schizophrenia in Kazakhs. An association of early-onset schizophrenia with genetic markers in three genes (*VRK2*, *KCNB2*, and *CPVL*) was found. An association of rs2312147 in the *VRK2* gene with schizophrenia was also previously reported in the Chinese population, so this marker may be considered as possibly race-specific. Two groups consisting of four and six genes demonstrating intergenic epistatic interactions were revealed by multifactor dimensionality reduction methods. The gene ontologies of 14 studied genes were reduced to variants of one molecular function (peptidase activity) and one biological process (positive regulation of biosynthesis processes). Bioinformatic analysis of the protein–protein interactions of products of the genes under study demonstrates that the products of six out of 14 genes may be involved in a single interrelated network, the major connecting link of which is represented by their ubiquitination by the UBC protein.

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INTRODUCTION

In recent years, schizophrenia remains one of the most widely studied mental illnesses; the heterogeneity of its etiology and clinical manifestations is undeniable. According to WHO, this disorder is a leader in the list of pathological states that lead to the disability and social exclusion of patients. Research aimed at studying the molecular-genetic aspects of neuropsychiatric diseases have shown that schizophrenia is a typical multifactorial disease (MD), the development of which is the consequence of a combination of intergenic and gene–environmental influences and interactions [1–3].

Genome-wide associations studies (GWAS) are one of the main approaches to the identification of the genetic components of MDs in recent years. An important component of the search for genetic components of MDs is replicative studies. All of the markers detected in GWAS or genome-wide linkage studies (GWLS) need to be replicated on independent samples. At the moment, according to the database of published genome-wide association studies supported by the U.S. National Human Genome Research Institute (NHGRI), there are 31 published genome-wide schizophrenia studies, which were conducted from 2007 to 2013 [4] (data for May 10, 2014). These studies revealed more than 130 single nucleotide markers (SNPs) that were highly significantly (with $p < 1 \times 10^{-5}$) associated with the disease.

One of the problems of interpreting and applying the results of association studies is the population specificity of gene–trait associations [5]. The problem lies in the fact that the stable combinations of genetic variants (usually, common variants of polymorphisms) underlying the development of complex multifactorial diseases in humans are formed in the context of the gene pool structure and linkage disequilibrium prevailing in a particular population during the action of microevolution factors [6]. Thus, combinations of markers of predisposition to MDs, is recorded as a marker–trait association, just as the power of the effect of individual markers may differ in populations of different ethnic origins. The vast majority of GWAS on schizophrenia, as well as for any other MD, were carried out on Caucasoid populations. Out of the 31 genome-wide schizophrenia studies published up to the present, 24 were carried out only on Europeoids, one work compared African Americans to White Americans [7], and six works addressed populations of East Asian Mongoloids (Chinese and Japanese) [8–13].

Thus, replicative research of genetic markers of schizophrenia identified in GWAS on populations of different ethnic origins seems to be an urgent task in the search for markers of the disease and identification of the pattern of population specificity of associations.

Schizophrenia is a clinically heterogeneous disease. One form of the disease is early-onset schizophrenia. The ambiguous attitude of a number of psychiatric schools regarding the very fact of the existence of childhood schizophrenia requires further research in terms of the age-comparative aspect. As noted by almost all researchers, difficulties in the timely diagnosis of schizophrenia are largely dependent on the fact that its debut often falls in childhood or adolescence—periods in which it is extremely difficult to distinguish between the initial symptoms of the disease and premorbid characteristics. The age pathomorphosis of childhood schizophrenia is characterized, in particular, by the fact that a mental defect, which forms together with schizophrenia (though having a more complex structure), is coupled with a child development violation concomitant to the process (disontogenesis), which may hinder early diagnosis of this disease. As in other age groups, early childhood schizophrenia runs continuously (malignantly and sluggishly) and episodically (in terms of the progredience degree, it is closer to the malignant type, with medium and low progredience degrees) [14, 15]. In cases of the debut of schizophrenia in adolescence (often during active puberty), we observed paranoid and attack-like progredient schizophrenia.

Studies on the search for genetic markers of early forms of schizophrenia are scarce [16–20]. All of them are part of the strategy of known candidate genes. From the viewpoint of genetic mapping of MDs, the forms of the disease with early onset and a more severe course are the preferred targets for association studies, as they are usually characterized by a more pronounced contribution of the genetic component [21].

The objective of this work was to find genetic markers of schizophrenia with onset in childhood and adolescence in Kazakhs based on replicative analysis of markers identified in genome-wide studies of schizophrenia and its cognitive endophenotypes.

MATERIALS AND METHODS

We examined children and adolescents with schizophrenia, as well as adults (with the beginning of the endogenous process in childhood), who were treated in the clinical departments of the Republican Scientific and Practical Center for Psychiatry, Psychotherapy and Addiction of the Ministry of Health of the Republic of Kazakhstan (the clinical base of the Department of Psychiatry, Psychotherapy, and Addiction of KazNMU) in the branches of the City Mental Health Center of Almaty, as well as children and adolescents with initial manifestations of schizophrenia from the group of outpatient care and the contingent of the specialized institution for psychochronics in the city of Almaty.

The sample consisted of 112 patients of Kazakh ethnicity aged from 4 to 30 years. The average age in the group of patients at the time of the survey was 16.8 years. As the basis for the formation of groups of probands, the present study has taken the age of the onset of initial symptoms and the age of the debut of schizophrenia in childhood and adolescence. Diagnoses of patients matched the headings of ICD-10: early childhood schizophrenia (F20.8xx3 in ICD-10 code)—58 patients, paranoid schizophrenia with continuous flow pattern (F20.00) and attack-like progredient schizophrenia (F20.01; F20.02)—54 patients.

The control group consisted of 190 individuals of Kazakh ethnicity, aged from 19 to 76 years, with no history of neuropsychiatric diseases. The average age in the control group was 31.6 years. The older age group was chosen in order to avoid inclusion of the patients whose disease has not yet manifested itself or was not diagnosed in time. The ethnicity of patients and members of the control group was established on the basis of a survey, and the study included those individuals who did not crossbreed in three generations. The study was approved by the bioethical committee of the Federal Agency of Scientific Organizations of Russia. All of the probands (their legal representatives) and donors (individuals in the control group) gave their informed consent for research and collection of peripheral blood.

For replication analysis of associations, we selected 15 single nucleotide genetic markers for which we found a highly reliable association with schizophrenia and with cognitive characteristics that are endophenotypes of this disease in recent GWAS [22–28]. The characteristics of the genes and genetic markers are shown in Table 1.

The DNA was isolated by phenol-chloroform extraction from whole venous blood. Genotyping was carried out by PCR in real time on TaqMan-samples produced by Applied Biosystems (United States) according to the manufacturer's protocol on a thermocycler with real-time PCR detection produced by Bio-Rad (United States).

Testing for the Hardy–Weinberg equilibrium (HWE) and calculation of expected heterozygosity was carried out by standard methods of population biometrics. Comparison of allele and genotype frequencies in the groups was carried out with the maximum likelihood criterion χ^2 (ML χ^2). The strength of associations was evaluated in values of the odds ratio OR and its 95% confidence interval (95%CI). Analysis of intergenic and gene–environment interactions was carried out by reducing the multidimensional dimensionality in the MDR (Multifactor Dimensionality Reduction, http://www.multifactordimensionalityreduction.org) program. For analysis of biological pro-

Table 1. Markers and genes for replication analysis of associations with schizophrenia

No.	SNP	Minor allele	Gene
1	rs1502844	C	*SLCO6A1*, family of solute organic anion carrier, element 6A1
2	rs9960767	C	*TCF4*, transcription factor 4
3	rs2312147	T	*VRK2*, vaccinia kinase 2
4	rs3131296	T	*NOTCH4*, neurogenic locus, notch protein homolog 4
5	rs12807809	C	*NRGN*, neurogranin
6	rs1572299	C	Intergenic region: *TLR4*, toll-like receptor type 4 and *DBC1*, protein 1 deleted in bladder cancer
7	rs17594526	T	*TCF4*, transcription factor 4
8	rs1344706	C	*ZNF804A*, zinc finger protein 804A
9	rs16977195	G	*AGBL1*, ATP/GTP binding protein 1, cytosolic carboxypeptidase
10	rs7341475	A	*RELN*, reelin
11	rs8020441	G	*ZFP64P1*, zinc finger protein 64, pseudogene 1 homolog in mice
12	rs2247572	T	*KCNB2*, potential-dependent potassium channel, Shab-related subfamily, element 2
13	rs2616984	G	*CSMD1* (*KIAA1890*), CUB and Sushi multiple domain 1
14	rs2229741	T	*NRIP1,* protein 1 interacting with nuclear receptor
15	rs2252521	T	*CPVL*, vitellogenin-like carboxypeptidase

cesses associated with the studied genes and gene networks, we used the bioinformatics resources DAVID (The Database for Annotation, Visualization and Integrated Discovery), KEGG (Kyoto Encyclopedia of Genes and Genomes), and STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) [29–31].

RESULTS

Frequencies of Genes and Genotypes and Associations of Genetic Markers with Early-Onset Schizophrenia

The frequencies of genotypes and minor alleles, the expected heterozygosity, and the achieved significance level of compliance of the observed distribution of genotypes to one expected under the Hardy–Weinberg equilibrium in the patients and in the control are shown in Table 2. Out of the 30 investigated distributions of genotypes, we observed two deviations from the distributions expected under HWE for a single nucleotide polymorphism, rs8020441 of the *ZFP64P1* gene, in the patients with schizophrenia and for rs2252521 of the *CPVL* gene in the control sample. In general, the allelic frequencies in patients and in the control are similar and are in the range of variations observed in the world population according to the data of the HapMap Project and the 1000 Genomes Project [32, 33].

The comparison of allele frequencies and the calculation of odds ratios for disease development are shown in Table 3. For three out of the 15 SNPs, statistically significant associations were detected at the level of allele frequencies. The minor allele rs2252521 of *CPVL* (OR = 1.46, $p = 0.037$) and the major alleles rs2312147 of the *VRK2* gene (OR = 1.72, $p = 0.008$) and rs2247572 of the *KCNB2* gene (OR = 1.54, $p = 0.030$) were significantly more frequent among Kazakhs with early schizophrenia as compared to the control group. For two out of the three associated markers (SNPs of the genes *VRK2* and *KCNB2*), the association was also confirmed by significant differences in the distribution of genotypes estimated by the maximum likelihood criterion χ-square. For rs2252521 of the *CPVL* gene, the differences in the distribution of genotypes almost had a statistically significant character ($p = 0.092$). All three SNPs associated with early schizophrenia in the Kazakhs are noncoding nucleotide substitutions localized in introns.

Analysis of Intergenic Interactions

To analyze the intergenic interactions that influence a phenotypic characteristic (the presence of schizophrenia), we used multifactor dimensionality reduction (MDR) using an exhaustive search algorithm. As a result, eight possible combinations of cumulatively interacting loci were revealed. Two of the eight obtained combinations demonstrate high cross-validation consistency (CVC) (10 out of 10 of consistent cross-validation), which shows a significant effect of combinations of polymorphic markers of several loci on the phenotype.

The first set of cumulatively interacting polymorphism variants includes four SNPs (rs1502844, rs2312147, rs8020441, and rs2616984). The achieved level of significance (P) of the adequacy of the model of these four SNPs according to the test of signs is

Table 2. Distribution of genotypes and allele frequencies in patients and control group

No.	SNP	MA	Patients						Control					
			11	*12*	*22*	MAF	H_e	*P*	*11*	*12*	*22*	MAF	H_e	*P*
1	rs1502844	C	32	46	22	0.450	0.495	0.480	67	82	41	0.432	0.491	0.097
2	rs9960767	C	94	7	0	0.035	0.067	0.718	180	10	0	0.026	0.051	0.708
3	rs2312147	T	53	34	4	0.231	0.355	0.617	79	90	19	0.340	0.449	0.365
4	rs3131296	T	88	14	0	0.069	0.128	0.458	157	30	2	0.089	0.164	0.689
5	rs12807809	C	51	42	7	0.280	0.403	0.680	108	64	18	0.263	0.388	0.070
6	rs1572299	C	61	37	4	0.221	0.344	0.579	129	56	5	0.174	0.287	0.708
7	rs17594526	T	101	1	0	0.005	0.001	0.960	181	3	0	0.008	0.002	0.913
8	rs1344706	A	26	50	25	0.495	0.500	0.923	50	89	50	0.500	0.500	0.424
9	rs16977195	G	85	13	1	0.076	0.140	0.538	173	16	1	0.047	0.090	0.357
10	rs7341475	A	84	16	2	0.098	0.177	0.254	150	38	1	0.106	0.189	0.390
11	rs8020441	G	55	46	1	0.235	0.359	**0.011**	97	84	9	0.268	0.393	0.083
12	rs2247572	T	59	34	7	0.240	0.365	0.498	80	89	16	0.327	0.440	0.206
13	rs2616984	G	71	28	1	0.150	0.255	0.327	151	34	5	0.116	0.205	0.082
14	rs2229741	C	40	43	7	0.317	0.433	0.325	82	75	31	0.364	0.463	0.057
15	rs2252521	T	37	47	17	0.401	0.480	0.752	93	69	4	0.232	0.356	**0.032**

MA, minor (rare) allel. Quantities of genotypes: *11*, homozygotes for the frequent allele; *12*, heterozygotes; *22*, homozygotes for rare allele; MAF, minor allele frequency; H_e, expected heterozygosity; *P*, achieved significance level of matching of the observed distribution of genotypes to that expected under the Hardy–Weinberg equilibrium. Bold font indicates statistically significant deviations from HWE.

Table 3. Analysis of associations of genetic markers with schizophrenia in Kazakhs

No.	SNP	Gene	OR	95%CI	P_1	ML χ^2	P_2
1	rs1502844	*SLCO6A1*	1.08	0.75–1.54	0.671	0.33	0.846
2	rs9960767	*TCF4*	1.33	0.45–3.85	0.570	0.32	0.568
3	rs2312147	*VRK2*	0.58	0.38–0.89	**0.008**	7.58	**0.023**
4	rs3131296	*NOTCH4*	0.75	0.37–1.48	0.372	2.01	0.366
5	rs12807809	*NRGN*	1.09	0.73–1.63	0.664	2.11	0.349
6	rs1572299	*TLR4/DBC1*	1.35	0.86–2.10	0.168	1.97	0.374
7	rs17594526	*TCF4*	0.60	0.20–6.48	0.934	0.21	0.645
8	rs1344706	*ZNF804A*	0.98	0.69–1.40	0.910	0.17	0.920
9	rs16977195	*AGBL1*	1.65	0.77–3.52	0.163	1.79	0.409
10	rs7341475	*RELN*	0.92	0.50–1.67	0.768	2.03	0.363
11	rs8020441	*ZFP64P1*	0.84	0.55–1.27	0.382	3.45	0.178
12	rs2247572	*KCNB2*	0.65	0.43–0.98	**0.030**	6.66	**0.038**
13	rs2616984	*CSMD1*	1.38	0.81–2.35	0.204	3.80	0.150
14	rs2229741	*NRIP1*	0.81	0.54–1.20	0.270	4.62	0.099
15	rs2252521	*CPVL*	1.46	1.01–2.12	**0.037**	4.77	0.092

OR, odds ratio for the minor allele; P_1, the significance level of OR; ML χ^2, maximum likelihood of χ^2 for comparing the distribution of genotypes; P_2, level of significance. Bold font indicates significance levels <0.05.

0.011. The carrying accuracy of the training sample was 76.27% with a sensitivity of 89.15% and specificity of 63.39%. The carrying accuracy of the test samples (average reproducibility by 10 cross-validations) is equal to 62.29%, the sensitivity and specificity is equal to 70.00 and 56.58%, respectively. Thus, the first set of nonconsistent cross-validations consists of four interacting genes: *SLCO6A1, VRK2, ZFP64P1*, and *CSMD1*, while only one of them (*VRK2*) demonstrates a statistically significant association with schizophrenia in the Kazakhs at the level of individual SNPs.

The second set consists of six single nucleotide cumulatively acting polymorphisms; in addition to the above described four SNPs, it includes two polymorphic variants: rs17594526 of the *TCF4* gene and rs2229741 of the *NRIP1* gene. The carrying accuracy of the training and test samples for this set of markers totaled 90.55% (sensitivity of 98.95%, specificity of 82.14%) and 53.64% (sensitivity of 44.02%, specificity of 65.09%; $P = 0.377$). For this combination of markers, there is a weaker classification ability for 10 test samples despite the high carrying accuracy (>90%) of the training sample. Most likely, the problems of small sample sizes, which arise in multiple testing with an increasing number of subgroups with an increasing number of interacting genes in the training sample, lead to a decrease in sensitivity and loss of statistical significance of the model.

DISCUSSION

Functions and Roles of Genes That Showed Replicated Associations with Schizophrenia

In the present study, we found an association of early-onset schizophrenia in Kazakhs of the Republic of Kazakhstan with the markers of three genes (*VRK2*, *KCNB2*, and *CPVL*) that were previously found to have a highly significant correlation with the disease or its endophenotypes in genome-wide association studies. *VRK2* encodes a protein serine/threonine protein kinase that belongs to Group I of casein kinases. These casein kinases phosphorylate the hydroxyl group in serine or threonine residues. They are involved in the control of the cytoplasmic and nuclear processes, including the replication and repair of DNA, and are also involved in the control of apoptosis. *VRK2* is widely expressed in tissues of the human body, actively dividing cells (testis, leukocytes, fetal liver, and carcinomas). The possible role of *VRK2* in the susceptibility to schizophrenia, in our opinion, may be related to the fact that serine/threonine protein kinases bind the protein JIP1, which in neuronal cells acts as a factor of antiapoptosis in response to stress and plays a role in the development of the axon [34]. An association of *VRK2* markers with schizophrenia was found in one of the first GWAS [22] on Caucasoids. Later, the association of rs2312147 of this gene with the disease has been replicated on Chinese [35]. Independent replication of the association of this gene in Asian Mongoloids (Chinese and Kazakhs), perhaps, demonstrates the race-specific character of the association, which is probably due to the structural features of the gene pool or patterns of linkage disequilibrium at this locus in Mongoloids.

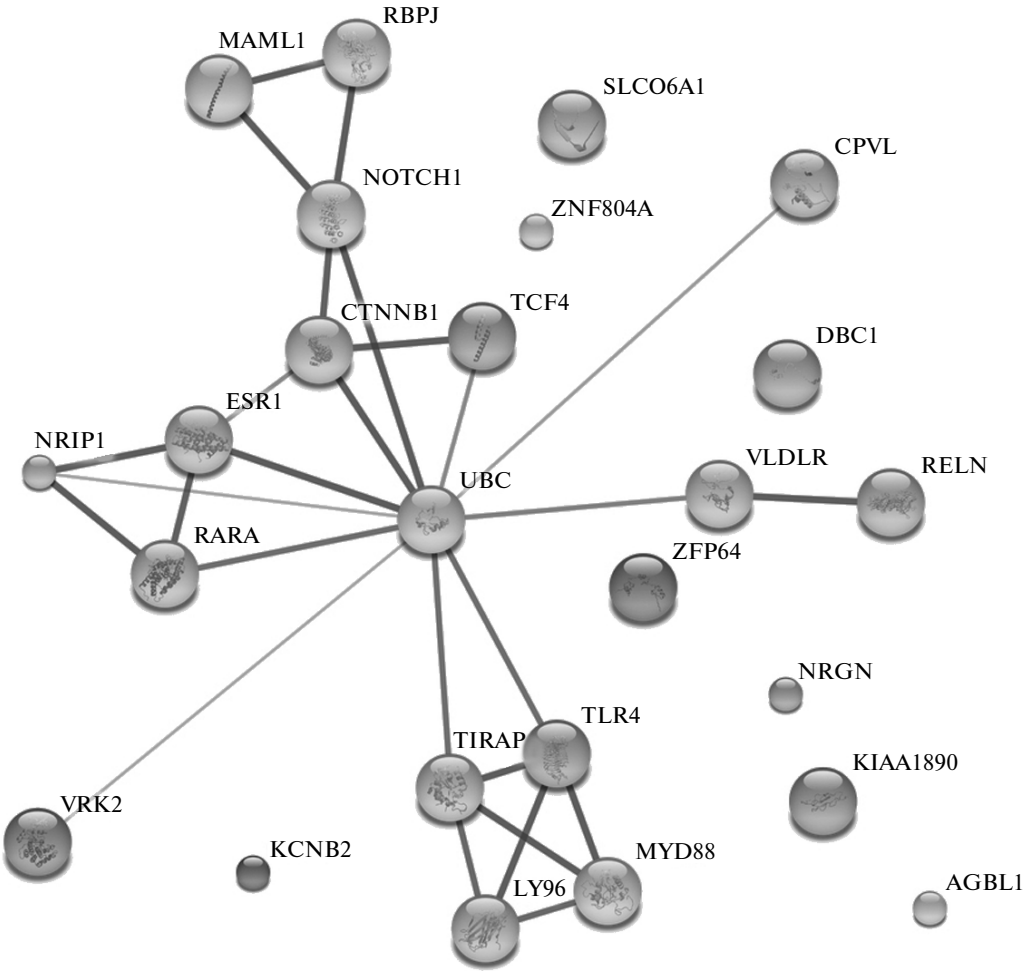
With respect to markers of the other two genes (*KCNB2* and *CPVL*), the association of which was replicated in the Kazakhs, the mechanisms of their possible involvement in susceptibility to the disease is even less clear. Markers of these genes have been identified in recent GWAS [26, 27] as those associated with cognitive abilities that are endophenotypes of schizophrenia. Replicative studies of these markers have not been carried out. *KCNB2* encodes a protein that is a part of the potential-dependent potassium channel. The gene is expressed in smooth muscle cells of the gastrointestinal tract. The protein encoded by *CPVL* is a carboxypeptidase and has strong sequence similarity with serine carboxypeptidases. It is possible that the association of markers in locus *CPVL* with the disease is not associated with this gene but rather with the closely located locus *LOC100506497*, which encodes miscRNA with an unknown function.

Biological Processes Associated with the Studied Genes and Gene Networks

Genome-wide association studies are carried out in the framework of the ideology of positional mapping and do not imply a functional relationship of the associated marker with the biological processes underlying the disease. Nevertheless, in the region marked by an associated polymorphic site, there must be genes or regulatory structures involved in metabolic, biochemical, or homeostatic systems. Violations of them often lead to development of the disease.

The 14 genes where the markers under study are localized (see Table 1) are not related by any common function or a single biological process. Functional annotation of these genes using the bioinformatics resource DAVID reveals seven gene ontologies (molecular functions and biological processes), in which a minimum of two of the studied genes are involved (Table 4). These ontologies are reduced to variants of a single molecular function (peptidase activity) and one biological process (positive regulation of biosynthesis). Peptidase activity is typical for the products of three genes (*AGBL1, RELN,* and *CPVL*), and biosynthesis regulation involves proteins encoded by three different genes (*TLR4, TCF4,* and *NRIP1*). Another eight genes did not show evidence of the generality of biological functions.

With the resource STRING, analysis of the protein–protein interactions of the products of the 14 genes under study shows that the products of six out of 14 genes may be involved in a single interconnected network, which also includes a number of other (not



Network of protein–protein interactions involving the products of 15 studied genes and 10 of their genes–partners. The size of the circle corresponds to the size of the protein. The thickness of the line between proteins reflects the confidence level of the association.

included in the present study) genes (figure). The proteins VRK2, CPVL, RELN, TLR4, NRIP1, and TCF4 are included in a single network, the central component of which is the protein ubiquitin C (UBC). The network is based on the analysis of experimental data, coexpression, databases and other evidence without using the "text mining" tool, which analyzes the coincidence of genes or their products in the same paragraph. All the six of these proteins, as well as NOTCH1, the *NOTCH4* gene homolog product, are capable of binding to ubiquitin C. Evidence for UBC association with these proteins was obtained in experimental studies using different approaches of protein chemistry [36–40]. It is noteworthy that the products of two of the three genes having associations with schizophrenia replicated in the present study (*VRK2* and *CPVL*) are also included in the network of ubiquitin-binding proteins.

Ubiquitin C in a monomeric or polymeric form is associated with a wide range of proteins and peptides through various lysine residues of polyubiquitin chains. The latter, binding themselves to target proteins, are involved in performing various functions, including cell cycle regulation, lysosomal degradation, destruction of proteins, modification of kinases, endocytosis, and activation of transcription factors. Free ubiquitin is also involved in the activation of protein kinases and signaling. Perhaps, the functions of VRK2 as a protein kinase and CPVL as carboxypeptidase are modified or regulated by the contact of proteins with ubiquitin C, and it is this that provides their network interactions in the pathogenesis of schizophrenia. It is also interesting that tbe products of three genes that show the effects of epistasis in the analysis by multifactor dimensionality reduction (MDR), *VRK2*, *TCF4*, and *NRIP1*, are also included in the network of ubiquitin-binding proteins.

Thus, in the present work we for the first time conduct an analysis of 15 single nucleotide replicative markers associated with schizophrenia and its endophenotypes according to GWAS in Kazakhs. This study is the first work to search for genetic markers of

Table 4. Gene ontologies of 14 genes, in the region of which the studied markers are localized

Code according to Gene Ontology	Molecular function (MF) or biological process (BP)	Involved genes	*P*
GO:0004180	Carboxypeptidase activity (MF)	*AGBL1, CPVL*	0.028
GO:0008238	Exopeptidase activity (MF)	*AGBL1, CPVL*	0.058
GO:0070011	Peptidase activity acting on L-amino acid peptide (MF)	*AGBL1, RELN, CPVL*	0.064
GO:0010557	Upregulation of biosynthesis of macromolecules (BP)	*TLR4, TCF4, NRIP1*	0.067
GO:0008233	Peptidase activity (MF)	*AGBL1, RELN, CPVL*	0.069
GO:0031328	Upregulation of cellular biosynthetic processes (BP)	*TLR4, TCF4, NRIP1*	0.073
GO:0009891	Upregulation of biosynthetic processes (BP)	*TLR4, TCF4, NRIP1*	0.075

P, significance level of the modified Fisher's exact test showing the randomness of the enrichment of the ontology by the studied genes.

early-onset schizophrenia in Mongoloid populations. Despite the relatively small sample of patients, which is due to the rarity of the studied form of schizophrenia, we found a statistically significant association for three genetic markers with the disease (SNP in genes *VRK2*, *KCNB2*, and *CPVL*). An association of rs2312147 of *VRK2* with schizophrenia was also previously replicated in the Chinese, which allows us to consider this marker as possibly race-specific. By reducing the dimensionality of multifactorial data, we have found two groups of four and six genes showing intergenic epistatic interactions. Analysis of the functions of the studied genes, biological processes, and gene networks suggested that the possible involvement of the protein VRK2 in the molecular mechanisms of schizophrenia may be associated with its protein kinase activity in the tissues of the nervous system and that the general process that connects the genes under study into a single network may be ubiquitylation of their products by the UBC protein. Further study of the association of the genes under study with various forms of schizophrenia is of interest, since the features of psychopathology, the flow, and the dynamics of the formation of initial states of this process give rise to the assumption of heterogeneity of its etiopathogenesis and genetic component.

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