Over-expression of dopamine D$_2$ receptor and inwardly rectifying potassium channel genes in drug-naive schizophrenic peripheral blood lymphocytes as potential diagnostic markers

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Abstract. Schizophrenia is one of the most common neuropsychiatric disorders affecting nearly 1% of the human population. Current diagnosis of schizophrenia is based on complex clinical symptoms. The use of easily detectable peripheral molecular markers could substantially help the diagnosis of psychiatric disorders. Recent studies showed that peripheral blood lymphocytes (PBL) express subtypes of D1 and D2 subclasses of dopamine receptors. Recently, dopamine receptor D$_3$ (DRD3) was found to be over-expressed in schizophrenic PBL and proposed to be a diagnostic and follow-up marker for schizophrenia. In this study we screened PBL of 13 drug-naive/drug-free schizophrenic patients to identify additional markers of schizophrenia. One of the benefits of our study is the use of blood samples of non-medicated, drug-naive patients. This excludes the possibility that changes detected in gene expression levels might be attributed to the medication rather than to the disorder itself. Among others, genes for dopamine receptor D$_2$ (DRD2) and the inwardly rectifying potassium channel (Kir2.3) were found to be over-expressed in microarray analysis. Increased mRNA levels were confirmed by quantitative real-time PCR (QRT-PCR) using the SybrGreen method and dual labeled TaqMan probes. The use of both molecular markers allows a more rapid and precise prediction of schizophrenia and might help find the optimal medication for schizophrenic patients.

Keywords: Schizophrenia, lymphocyte, dopamine receptor D$_2$ (DRD2), inwardly rectifying potassium channel (Kir2.3), microarray, real-time PCR

1. Introduction

Schizophrenia is a severe neuropsychiatric disorder characterized by disturbances in cognitive, emo-tional and motor processes [18]. Dysfunction of the dopaminergic system is thought to be one of the main reasons for the development of this disease [1]. Expression of neurotransmitters, neuropeptides and the D2 subclass of dopamine receptor genes (D$_2$, D$_3$, D$_4$ subtypes) in immune cells, mainly in peripheral blood lymphocytes (PBL) has been reported earlier by several authors [6,7,20,31–33]. It has been suggested that these may reflect the status of the corre-

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sponding brain receptors. In recent years D_3 subtypes of D2 subclass of dopamine receptor genes (DRD3) were found to be up-regulated in schizophrenic PBL and proposed to be a useful peripheral marker for the identification and follow-up of schizophrenia [13,15]. Fuchs's group reported decreased mRNA level of $\alpha 7$ nicotinic acetylcholine receptor ($\alpha 7$AChR) gene in PBL of schizophrenic patients [28]. These expression analysis studies were based on either dopaminergic ligand binding assays or on RNA levels detected by quantitative RT-PCR.

In recent years, however, new molecular methods have been developed to analyze gene expression profiles either in a high throughput (microarray technology) or in a more precise way using quantitative real-time PCR (QRT-PCR). Several groups have reported gene expression changes in postmortem schizophrenic brain applying microarray technology [12,19,21–24]. Identification and use of multiple peripheral molecular genetic markers would be extremely helpful for the precise early diagnosis and the follow-up of schizophrenia and for the implementation of effective therapy. Therefore, the aim of this study was to identify genes with differential expression in schizophrenic PBL using both high-throughput microarray analysis and QRT-PCR technique. We screened exclusively drug-naive/drug-free schizophrenic patients, excluding the possibility of gene expression being affected by medication.

2. Materials and methods

2.1. Patients

Patients were recruited from the inpatient unit of the Department of Psychiatry, University of Szeged and informed consent was obtained after explanation of the study. Each patient went through profound medical examination including physical and neurological examination as well as routine laboratory urine drug tests. Each of them received the Mini International Neuropsychiatric Interview Plus (MINI Plus) [4], and was diagnosed with schizophrenia or schizophreniform psychosis according to DSM-IV [3]. Psychiatric symptoms were assessed using the Positive and Negative Syndrome Scale (PANSS) [14], the Clinical Global Impression (CGI) and the Global Assessment of Functioning (GAF) scale [3]. Two trained psychiatrists performed clinical ratings. The study was conducted in accordance with the Declaration of Helsinki. The demographical and medical data of 7 female and 6 male drug-naive or drug-free schizophrenic patients and 10 healthy control individuals involved in this study are listed in Table 1.

2.2. Isolation of PBL and RNA from schizophrenic patients and control individuals

The lymphocyte fraction of 10 ml of total blood (collected in EDTA-treated tubes) was washed 3 times with red blood lysis buffer (5 mM $MgCl_2$, 10 mM NaCl, 10 mM Tris-HCl, pH 7.0, Promega, Madison, WI, USA) to lyse erythrocytes. RNA isolation from the clear pellet of lymphocytes was carried out by the RNA isolation kit of Macherey-Nagel (Macherey-Nagel, Düren, Germany) according the manufacturer's instructions. RNA samples were stored at $-80°C$ in the presence 30 U of Prime RNAse inhibitor (Eppendorf, Hamburg, Germany). The quality and quantity of isolated RNA were checked by electrophoresis and spectrophotometry (NanoDrop, Rockland, DE, USA).

2.3. Microarrays, probe preparation and hybridization

1200 amplified cDNA inserts from the human lymphocyte cDNA Matchmaker library (Clontech, BD Biosciences Franklin Lakes, NJ, USA) and 2000 sequence-verified clones from a mixed human library in pBluescript SK II (−) plasmid (New England Biolabs, Hertfordshire, England) were amplified by PCR with plasmid-specific primers and purified with MultiScreen-PCR plate (Millipore, Billerica, MA, USA), resuspended in 50% dimethyl sulfoxide/water, and arrayed on FMB cDNA slides (Full Moon BioSystems, Sunnyvale, CA, USA) using a MicroGrid Total Array System (BioRobotics, Cambridge, UK) spotter. Post-processing and blocking of the microarrays were performed as described previously [30]. For probe preparation, 2 μg of total RNA (a pool of 14 schizophrenic patients) was reverse transcribed using poly-dT-primed Genisphere Expression Array 350 Detection Kit system (Genisphere, Hatfield, PA, USA) according the manufacturer's instructions. cDNA with capture sequence was hybridized onto a human cDNA microarray containing 3200 human cDNA clones. Both the first step, cDNA hybridization and the second step, capture reagent hybridization were carried out in a Ventana hybridization station (Ventana Discovery, Tucson, AZ, USA) using the "antibody" protocol. The first hybridization was performed at $40°C$ for 6 hours in "FGL2" hybridization buffer (10x Denhart solution, 0.25 M sodium phosphate buffer pH 7.0, 1 mM EDTA, 1x SSC, 0.5% SDS), then 2.5 μl each of Cy5 and Cy3 capture reagents in 200 μl "Chiphyb" hybridization buffer (Ventana) were added to the slides and incubated

Table 1
Demographic and medical data of schizophrenic patients

Patients	Status (duration of drug-free) period	Age	Duration of illness	PANSS				CGI	GAF	Medication on date of sampling		
				Positive	Negative	Global	S			APS	ANX	other
Male 2	F (1 month)	34	3 years	26	25	55	106	4	50	Ø	Ø	Ø
Male 3	N	34	6 months	25	21	36	82	4	40	Ø	Ø	Ø
Male 4	F (4 months)	36	4 years	33	31	65	129	6	25	Ø	Ø	Ø
Male 6	N	25	4.5 years	29	36	66	131	6	20	Ø	Ø	Ø
Male 7	N	25	7 months	34	32	74	140	6	20	Ø	Ø	Ø
Male 8	N	24	6 months	14	37	49	100	5	25	Ø	Ø	Ø
Female 1	F (3 months)	46	15 years	31	25	58	114	6	30	Ø	Ø	Ø
Female 2	F (3 months)	34	14 years	34	40	85	159	7	15	Ø	Ø	Ø
Female 3	F (1 year)	67	17 years	29	28	51	108	5	30	Ø	Ø	Ø
Female 4	F (1 year)	29	5 years	29	30	54	113	6	30	Ø	Ø	Ø
Female 5	N	27	9 months	40	34	81	155	7	15	Ø	Ø	Ø
Female 6	N	33	8 months	22	23	54	99	4	40	Ø	Ø	Ø
Female 8	N	23	3 months	22	28	62	112	5	30	Ø	Ø	Ø

N: drug-naive, F: drug-free; PANSS: Positive and Negative Syndrome Scale; positive: positive syndrome items; negative: negative syndrome items; global: global syndrome items; S: PANSS total score; CGI: Clinical Global Impression; GAF: Global Assessment of Functioning; APS: antipsychotic; ANX: anxiolytic.

at 42°C for 2 hours. After hybridization, the slides were washed twice in 0.2X SSC at RT for 10 min, then dried and scanned. Scanning and data analysis were done as described earlier [26].

2.4. Real-time PCR

For QRT-PCR, 1 μg samples of total RNA from individual schizophrenic patients and control individuals were reverse transcribed using SuperScript II RNase H- Reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The reactions were carried out in final volume of 20 μl in the presence of 3.5 μM Random Hexamer Primer, 1x First-Strand Buffer (250 mM Tris-HCl (pH 8.3) 375 mM KCl, 15 mM MgCl$_2$), 500 nM dNTP mix (500 nM of each) 10 mM DTT, 40 U Prime RNAse inhibitor (Eppendorf) and 200 U of SuperScript II RNase H- Reverse transcriptase (Invitrogen) at 42 °C for 2 hours. RNA and the random primer mixture were denatured at 65°C for 5 minutes before the RT reaction. The enzyme reaction was terminated by inactivating reverse transcriptase at 70°C for 15 minutes. The RT reaction mixture was next diluted 4 times with sterile water. 1 μl of the diluted reaction mix was used for QRT-PCR using either SybrGreen dye or TaqMan probes. Reactions using SybrGreen protocol were done with ABsolute QPCR SYBR Green mix (ABGene, Surrey, UK) in a RotorGene real-time Q-PCR machine (Corbett Research, Mortlake, Australia) according to manufacturer's instructions at a final primer concentration of 150 nM under the following conditions; 15 min at 95°C, 45 cycles of 95°C for 15 sec, 60°C for 25 sec and 72°C for 25 sec. The fluorescence intensity of SybrGreen dye was detected after each amplification step. Melting temperature analysis was done after each reaction to check the quality of the reaction. PCR reactions with TaqMan probes were done using ABsolute QPCR mix (ABGene) according to manufacturer's instructions in the presence of 300 nM of primers and 250 nM of TaqMan probes under the following conditions: 15 min at 95°C and 45 cycles of 95°C for 15 sec, 60°C for 1 min. Fluorescein dye (FAM) intensity was detected after each cycle. PCR primers and TaqMan probes were designed using the Primer Express Software (Applied Biosystems, Foster City, CA, USA). Primers and TaqMan probes used in this study are summarized in Table 2. Dual-labeled probes were prepared by Bioneer (Daejeon, Korea) with Fluorescein dye at the 5' end and Dabsyl dye at the 3' end. The quality of the primers was verified by MS analysis provided by Bioneer. A non-template control sample was used for each PCR run to check the genomic DNA contaminations of the cDNA template. The results were analyzed using the Pfaffl method [29], which provides correction for the differences between the amplification efficiencies of the reactions.

2.5. Statistical analysis

The p value was determined by the unpaired Student's t test (two-tailed). To divide the gene expression dataset into groups of similar observations, agglomerative hierarchical clustering was applied. The method employed was the group average method. Euclidian metric (root sum-of-squares of differences) was used to calculate the dissimilarities between observa-

Table 2
List of primer and TaqMan probe sequences used for quantitative real-time PCR

| Short name	PCR primers	TaqMan probe	Gene name	Acc. No.
DRD2	For CTGCTCATCGCTGTCATCGT Rev CTCGCGGGACACAGCC	TCGGCAACGTGCTGGTGTGCA	Human D2 dopamine receptor	X51362
Kir2.3	For CTTCCCCCACTGACTCTTCAAG Rev CCAGCAGTCCAGCCACCTT	TGCCCTCTTTGCTCTCAGAACCTTGG	*Homo sapiens* potassium inwardly-rectifying channel (Kir2.3)	NM_004981
Actin	For TCACCGAGCGCGGCT Rev TAATGTCACGCACGATTTCCC	CAGCTTCACCACCACGGCCGA	Human actin, beta (ACTB),	NM_001101
HPRT	For TGACACTGGCAAAACAATGCA Rev GCTTGCGACCTTGACCATCT	CTTTGCTTTCCTTGGTCAGGCAGTAT-AATCCA	Human hypoxanthine phosphoribosyltransferase (HPRT)	M31642
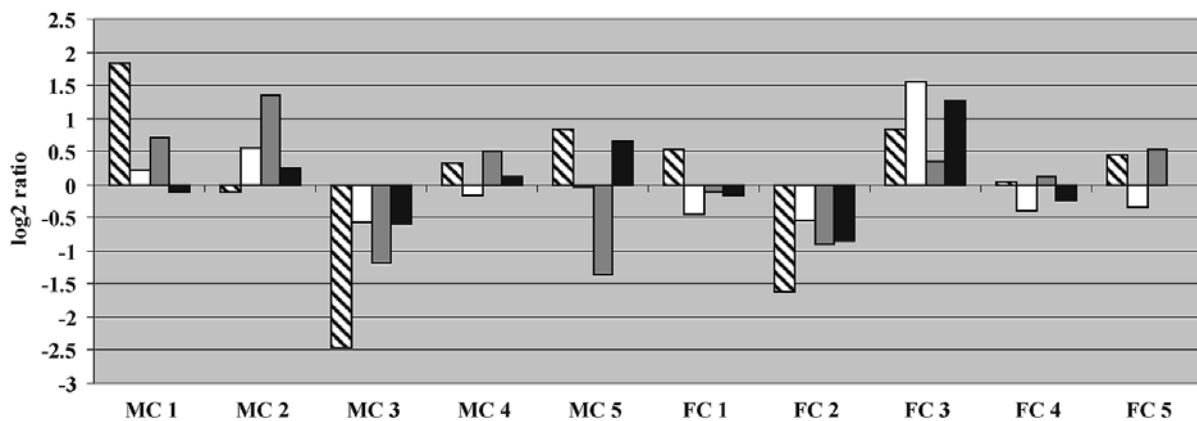
Fig. 1. Variation of the relative expression levels of DRD2 and Kir2.3 genes in non-affected control samples. Each individual Ct values of both genes were normalized to internal control genes. These normalized Ct values in all individual samples were compared to the average normalized Ct values of the 5 corresponding sex matched control samples. Relative expression levels are plotted as log2 ratios on the Y-axis. Dashed bars and white bars represent Kir2.3 gene expression levels analyzed by SybrGreen and TaqMan protocols, respectively. Grey and black bars represent the DRD2 gene expression level analyzed by SybrGreen and TaqMan protocols, respectively. (MC, male control; FC, female control).

tions. The routine is implemented in the R1.8.1 statistical software.

3. Results

We screened 13 drug-naive or drug-free schizophrenic patients (7 female and 6 male, age range 23–67 years, average 34 ± 12) and 10 control individuals (5 male and 5 female, age range 28–65 years, average 38 ± 10) to identify novel peripheral genetic markers of schizophrenia. Demographic data of schizophrenic patients and control individuals are summarized in Table 1. None of either the affected or the non-affected individuals was under antipsychotic treatment or other medication.

cDNA microarray analysis was performed in order to pre-screen the expression pattern of schizophrenic PBL and to identify potential candidate peripheral marker genes. Total RNA pools of 13 affected male and female patients and 10 control individuals were reverse transcribed and hybridized onto human cDNA microarray containing 3200 human cDNA clones. Positive

clones showing more than two-fold changes in their expression as compared to controls were sequenced. 2.56 ± 0.26- and 6.75 ± 2.3-fold increase in expression level of DRD2 and Kir2.3 was detected, respectively, in schizophrenic patients as compared to healthy individuals (Fig. 2). Based on these preliminary data and considering the potential role of these genes in the pathogenesis of schizophrenia, the expression levels of DRD2 and Kir2.3 genes were subjected to further analysis using QRT-PCR with SybrGreen dye and dual labeled TaqMan probes.

After reverse transcription of the individual total RNA samples of 13 affected and 10 non-affected individuals, QRT-PCR was performed on cDNA templates in order to determine the expression levels of DRD2 and Kir2.3 genes. β-actin or hypoxanthine phosphoribosyltransferase (HPRT) genes were used as internal controls. Relative quantification using Ct values and reaction efficiency values was performed to determine the exact ratio. Each individual Ct values of both genes were normalized to internal control genes. These normalized Ct values in all individual samples were compared to the average normalized Ct values of the 5
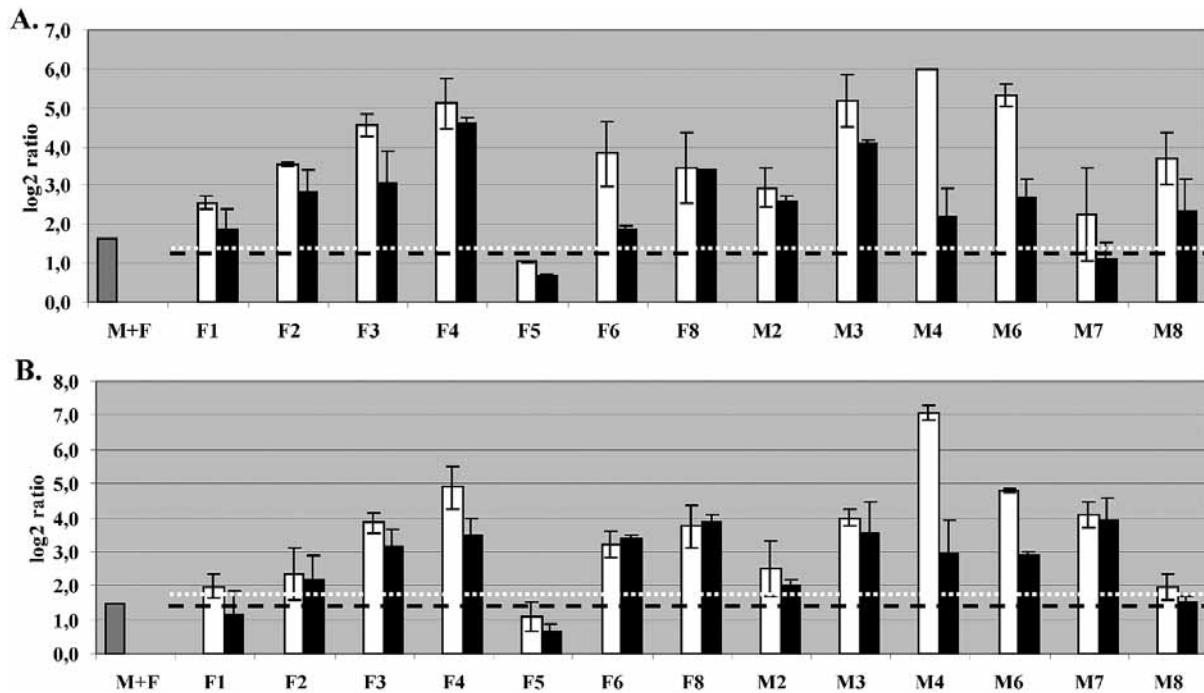
Fig. 2. Relative expression levels of DRD2 (A) and Kir2.3 (B) genes in schizophrenic samples as ratios as compared to the healthy controls. Each individual Ct values of both genes were normalized to internal control genes. These normalized Ct values in all individual samples were compared to the average normalized Ct values of the 5 corresponding sex matched control samples. Ratios are shown as log2 ratios on the Y-axis. Grey bars show the relative expression level of DRD2 and Kir2.3 gene analyzed by microarray method. White and black bars represent data analyzed by QRT-PCR using SybrGreen dye and TaqMan protocols, respectively. (A) White dotted line at (log2 ratio = 1.4) and black dashed line at (log2 ratio = 1.3) show the background expression level of DRD2. (B) White dotted line at (log2 ratio = 1.8), and black dashed line at (log2 ratio = 1.5) show the background expression level of Kir2.3. Calculation of the value of background relative expression level is based on the variation of the relative expression levels of DRD2 and Kir2.3 genes in non-affected control samples. (M, male patient; F, female patient).

corresponding sex matched control samples using the Pfaffl method [29].

To determine the variation of DRD2 and Kir2.3 mRNA levels in the non-affected samples, the relative expression ratios of both genes in all individual healthy samples were calculated as follows. Normalized Ct values of each individual healthy sample were compared to the average normalized Ct values of the 5 corresponding sex matched control samples. The variation of relative expression level of DRD2 and Kir2.3 genes in control individuals is shown in Fig. 1. Relative mRNA levels of DRD2 and Kir2.3 in the non-affected samples varied in the range of 0.39- to 2.56-fold and 0.18- to 3.54-fold, respectively, using the SybrGreen method and 0.55- to 2.42-fold and 0.67- to 2.92-fold, respectively, using the TaqMan probe method. Considering these variations of relative expression levels of DRD2 and Kir2.3 genes in the non-affected control samples, only affected PBLs with relative expression levels of DRD2 and Kir2.3 higher than any of the non-affected control were regarded as positives. Us-

ing the SybrGreen method, ratios above 2.56 and 3.54 were regarded as up-regulated in the case of DRD2 and Kir2.3 genes, respectively. In the case of the TaqMan protocol, ratios above 2.42 and 2.92 were regarded as up-regulated in the case of DRD2 and Kir2.3 genes, respectively (Fig. 2, white dotted and black dashed lines).

The DRD2 gene was found to be significantly over-expressed in 6 male and 6 female schizophrenic patients as compared to healthy controls in the range of 5.7-fold to 64.7-fold using the SybrGreen method. The relative expression level of the DRD2 gene of one female affected PBL (F5) was below background level. In this case a 2.1-fold over-expression could be detected. The average increase in DRD2 gene expression detected by the SybrGreen method was 21.4 ± 18.5-fold ($p = 0.0019$) (Fig. 2(A), white bars). Using the TaqMan probe, 6 female and 5 male schizophrenic PBLs showed increased DRD2 mRNA levels (3.7- to 24.1-fold). The relative expression levels of DRD2 of one female and one male affected PBLs (F5, M7) were below the calculated background level (1.6- and

2.2-fold, respectively). The average increase in DRD2 gene expression determined by TaqMan protocol was 7.8 ± 6.3-fold ($p = 0.0023$) (Fig. 2(A), black bars).

In the case of 6 male and 6 female schizophrenic PBL, the expression level of Kir2.3 was also above the background expression level as compared to healthy controls. The ratios of Kir2.3 mRNA levels in affected versus non-affected samples varied in the range of 4- to 133.6-fold using the SybrGreen method. The relative expression level of the Kir2.3 gene of one female affected PBL (F5) was below the background value (2.2-fold). The average increase in the relative expression level of Kir2.3 determined by the SybrGreen method was 22 ± 34.7-fold ($p = 0.0535$) (Fig. 2(B), white bars). These changes in expression level were also confirmed by the method using dual labeled TaqMan probes. 5 female and 6 male schizophrenic PBL showed increased Kir2.3 mRNA levels (2.8- to 15.8-fold). In the case of 2 female affected PBLs (F1, F5), the relative expression levels of Kir2.3 were below the calculated background level (2.4- and 1.6-fold, respectively). The average increase in Kir2.3 gene expression determined by TaqMan probe was 8.2 ± 4.8-fold ($p = 0.0001$) (Fig. 2(B), black bars). Differences in expression levels observed by the two different real-time PCR methods could be explained by the higher precision and sensitivity of the TaqMan protocol.

To determine similarity groups within gene expression dataset, agglomerative hierarchical clustering was applied. The non-affected control and the affected samples were correctly classified into two separate clusters with the evident exception of the schizophrenic sample F5. There were no significant differences between the relative expression levels of female and male patients (Fig. 3).

4. Discussion

In the present study we showed for the first time that both DRD2 and Kir2.3 genes are over-expressed in drug-naive and drug-free schizophrenic PBL as compared to healthy individuals using either microarray or quantitative real-time PCR method. One of the benefits of our study is the use of blood samples from non-medicated, drug-naive patients. This excludes the possibility, that changes detected in gene expression levels might be attributed to the medication instead of the disorder itself.

One of the hypotheses explaining the nature of schizophrenia is based on dysfunction of dopaminergic system, especially an increased occupancy of D2 subclass of dopamine receptors (DR) by dopamine in schizophrenic patients [1]. Antipsychotic drugs presently used for the treatment of schizophrenia act as antagonists of D2 subclass of DRs [35]. Since dopamine interacts directly with DRD3 and DRD2 on T cells and activates integrin-mediated T cell adhesion, over-expression of these genes in T cells can serve not only as a passive diagnostic marker but can also reflect a dynamic functional interaction [17]. Increased expression of other representatives of D2 subclass of DRs, like the DRD3 gene in schizophrenic PBL has already been reported by other groups [13,15]. Medication, either typical or atypical had no effect on DRD3 expression level and medicated patients exhibited the same expression pattern. The expression level of DRD4 receptor genes was unchanged [13]. The correlation between DRD2 mRNA levels in different brain regions and in PBL has also been demonstrated in other diseases such as Alzheimer's dementia [5].

DRD2 belongs to D2 subclass of DRs and is coupled to a G-protein with an inhibitory effect on adenylyl cyclase activity [2]. Receptor-activated G-proteins can either activate (Kir3.1) or inactivate inwardly rectifying potassium channels (Kir2.3) [8,25]. Several different potassium channels are known to be involved in electrical signaling in the nervous system [9]. Kir2.3 is a member of the Kir2 family of constitutively active inward rectifier K^+ channels carrying large inward and small outward potassium currents [27]. This asymmetry in potassium ion conductance plays a key role in the excitability of muscle cells and neurons. Dopamine (DA) directly activates, through D2 subclass of DRs, an IAP (Islet activating protein, pertussis toxin) -sensitive G protein coupled with inward rectifier potassium channels in rat substantia nigra neurons [16,37], demonstrated that DRD2 and another inwardly rectifying potassium channel Kir3 form a stable functional complex both *in vitro* and *in vivo*. Gorelova et al. showed by whole cell patch-clamp study that DA depolarizes fast-spiking interneurons by suppressing an inwardly rectifying K^+ current via unknown DA mechanisms [10]. In an investigation of medial prefrontal cortex pyramidal neurons, Dong et al. showed that DA has a strong modulating effect on inwardly rectifying potassium currents (IRKC) [11]. They found that stimulation of both D1 and D2 subclasses of dopamine receptors produced inhibition of IRKC. These results suggest a strong association between DRs and inwardly rectifying potassium channels.

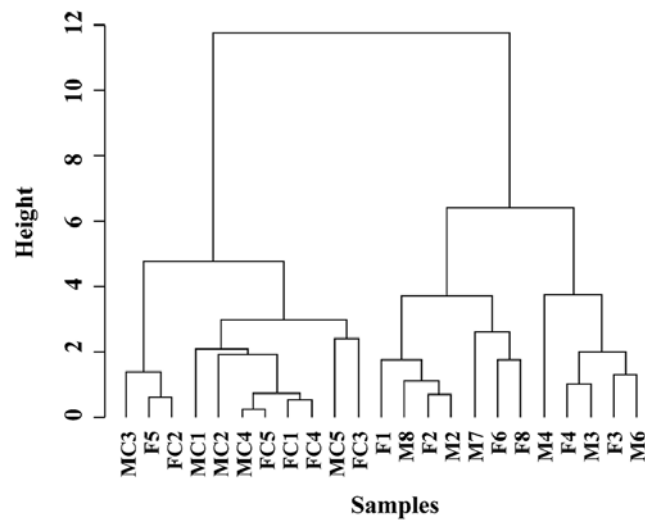The increased expression level of DRD2 gene in PBL is in agreement with other reports analyzing DRD2

Fig. 3. Hierarchical clustering based on expression dataset clearly separates into two main clusters: the non-affected control and affected samples with the evident exception of schizophrenic sample F5. (MC, male control; FC, female control; M, male patient; F, female patient).

mRNA levels in different brain regions. Using a quantitative RT-PCR technique, Roberts et al. detected increased expression levels for both isoforms of the DRD2 gene in some brain regions in schizophrenics as compared to controls [34]. Tallerico et al. measured elevated mRNA level of DRD2$_{longer}$ isoform using quantitative competitive RT-PCR in schizophrenic post-mortem frontal cortex regions in both medicated and non-medicated patients [36]. Vawter et al. screened the PBLs of five individual family members with schizophrenia comparing them to unaffected individuals in order to identify differentially expressed genes using microarray of 1128 brain related genes, but neither DRD2 nor Kir2.3 was presented on the microarray they used [38].

5. Conclusion

Based on the results of the present study, it seems that the already reported central increase of DRD2 expression is reflected in PBL as well. Among the genes that were found to be differentially expressed in affected PBL by microarray method, up-regulation in the expression of two genes, DRD2 and Kir2.3 was confirmed by SybrGreen and TaqMan QRT-PCR protocols. Since neither the affected nor the non-affected individuals were under antipsychotic treatment or other medication, elevated DRD2 and Kir2.3 mRNA levels reflect the disorder itself rather than the effect of medication. Our results suggest that DRD2 and Kir2.3 can serve as

additional peripheral genetic markers for the prediction of schizophrenia. To confirm these genes as potential markers, expression levels of further affected PBLs need to be analyzed. High-throughput analysis of already reported molecular markers and genes in combination with the ones presented here might promote the earlier and more precise diagnosis of this disorder. Diagnostic tests based on these results can be developed and help manage schizophrenia from the onset all the way to the chronic state.

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