Sequencing of five left–right cerebral asymmetry genes in a cohort of schizophrenia and schizotypal disorder patients from Russia

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Objective **Schizophrenia is a severe psychiatric disorder, affecting ~1% of the human population. The genetic contribution to schizophrenia is significant, but the genetics are complex and many aspects of brain functioning, from neural development to synapse structure, seem to be involved in the pathogenesis. A novel way to study the molecular causes of schizophrenia is to study the genetics of left–right (LR) brain asymmetry, the disease feature often observed in schizophrenic patients.**

Methods **In this study, we analyzed by sequencing five candidate LR cerebral asymmetry genes in a cohort of 95 schizophrenia and schizotypal disorder patients from Saint Petersburg, Russia. The gene list included *LMO4*, *LRRTM1*, *FOXP2*, the *PCDH11X/Y* gene pair, and *SRY*.**

Results **We found 17 previously unreported variants in the genes *LRRTM1*, *FOXP2*, *LMO4*, and *PCDH11X* in the 3′-UTR and 5′-UTR. The variants might contribute toward an altered mRNA processing, which could lead to altered mRNA amounts in developing neurons of the brain and establishment of an incorrect LR asymmetry profile.**

Conclusion **This is the first study in which multiple candidate genes for cerebral LR asymmetry and schizophrenia have been analyzed by sequencing. The approach to study the genetics of schizophrenia from the perspective of an LR cerebral asymmetry disturbance deserves more attention. *Psychiatr Genet* 24:75–80** © **2014 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

Schizophrenia is a severe mental disorder, affecting ~1% of any human population (Freedman, 2003). It is characterized by 'positive' symptoms, including hallucinations, delusions, and disorganized speech and 'negative' symptoms, including social withdrawal and affective flattening. As a result, patients affected by schizophrenia often show dysfunction in work, interpersonal relationships, or self-care. The disorder has a complex clinical picture that can additionally vary within the same individual during his/her life course, leading to the notion of 'schizophrenias', rather than a single homogeneous disorder (Boshes *et al.*, 2012). Given the complex clinical picture, it is not surprising that the genetics of schizophrenia are non-Mendelian. Schizophrenia has an important genetic component, up to 80% of factors leading to pathogenesis being genetic (Sullivan *et al.*, 2003), and the genetic studies of schizophrenia are advancing, leading to the discovery of ~20 candidate genes, whose role was supported by multiple studies (Burmeister *et al.*, 2008; Tiwari *et al.*, 2010; Gejman *et al.*, 2011; Girard *et al.*, 2011; Rodriguez-Murillo *et al.*, 2012). Recently, apart from long-established linkage and association studies of genetics of schizophrenia, new approaches have emerged, studying de-novo copy number variations and point mutations by resequencing [reviewed in Rees *et al.* (2012) and Rodriguez-Murillo *et al.* (2012)]. These various studies discovered genes important mostly in neural development, neurotransmission, and immune system (Tiwari *et al.*, 2010). Indeed, a widely accepted view of schizophrenia is that of a neurodevelopmental disorder, affecting normal brain development during embryogenesis and childhood (Rapoport *et al.*, 2005; Fatemi and Folsom, 2009). An aberrant brain development apparently leads to the multiple structural and functional brain abnormalities observed in schizophrenic patients (Ross *et al.*, 2006; Schmitt *et al.*, 2011). One of the hallmarks of a schizophrenic brain is reduced or even reversed left–right (LR) asymmetry of the brain structures (Sommer *et al.*, 2001; Oertel-Knochel and Linden, 2011). This finding allowed to propose that genes important for the establishment of LR asymmetry of the human brain are implicated in schizophrenia (Crow *et al.*, 1989). The main difficulty in this direction

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of investigation of schizophrenia genetics is that the genes responsible for the establishment of LR asymmetry of the human brain are hardly known, and they might not be the same as the well-described genes that determine LR asymmetry of the viscera (Malashichev and Wassersug, 2004; Malashichev, 2006; Sun and Walsh, 2006; Corballis, 2009; Crow *et al.*, 2009). In particular, individuals with *situs inversus totalis* of the viscera do not always show in parallel a functional or a structural reversal of brain LR asymmetry [reviewed in Malashichev (2006) and Renteria (2012)]. Nevertheless, several candidates for the role of the genes determining cerebral asymmetry have been described in the literature. The goal of this study was to screen by sequencing five genes, described in the literature, with a possible role in the development of LR asymmetry of the human brain, in a cohort of individuals with a diagnosis of schizophrenia. Therefore, the working hypothesis was that these genes were mutated; the mutations led to altered LR asymmetry of the brain and, eventually, to schizophrenia in these patients.

Methods

Ethics statement

Written informed consent was obtained from all the participants. The participants have not had a compromised capacity/ability to consent because they are outpatients at an ambulatory care clinic and are not psychotic. The experiments comply with the current laws of the country (The Russian Federation) in which they were conducted, and were also approved by the responsible authorities at the institute (Faculty of Medicine, Saint Petersburg State University, Saint Petersburg, The Russian Federation) where the work has been carried out. In addition, the research was carried out in full compliance with the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Patients

The participants are outpatients at the Saint Petersburg State University psychiatric ambulatory care clinic; all of them reside in Saint Petersburg, Russia. The cohort of 95 patients was selected on the basis of the diagnostic criteria of The International Statistical Classification of Diseases and Related Health Problems, 10th Revision (ICD-10), Chapter V. See Supplementary Table, Supplemental digital content 1, *http://links.lww.com/PG/A93* for details on each patient and Table 1 for overall statistics of the cohort. We selected patients with the diagnosis of either paranoid, or simple, or hebephrenic form of schizophrenia; we also included schizotypal disorder patients in the cohort. The patients were also characterized using the expanded 24-item Brief Psychiatric Rating Scale (BPRS-24), which includes the following parameters: somatic concern, anxiety, depression, suicidality, guilt, hostility, elevated mood, grandiosity, suspiciousness, hallucinations, unusual thought content, bizarre behavior, self-neglect, disorientation, conceptual disorganization, blunted affect, emotional withdrawal, motor retardation, tension, uncooperativeness, excitement, distractibility, motor hyperactivity, and mannerism-posturing (Ventura *et al.*, 1993; Dingemans *et al.*, 1995). Each item is rated with a score: 1 – not present, 2 – very mild, 3 – mild, 4 – moderate, 5 – moderately severe, 6 – severe, and 7 – extremely severe; in Table 1 and in Supplementary Table, Supplemental digital content 1, *http://links.lww.com/PG/A93*, we present the total scores (the sum of scores) for the patients.

Genetic screening

After literature review, five candidate genes for LR asymmetry of the human brain were identified. This list is similar to the one proposed previously (Crow *et al.*, 2009), but incorporated an additional sex chromosome gene, *SRY*. The list includes *LRRTM1* (*Leucine Rich Repeat Transmembrane Neuronal 1*), associated with schizophrenia and handedness (Francks *et al.*, 2007); *FOXP2* (*Forkhead Box P2*), found to be mutated in a speech disorder and associated with functional asymmetry of the brain (Lai *et al.*, 2001; Ocklenburg *et al.*, 2013b); *LMO4* (*Lim Domain Only 4*), found to be consistently asymmetrically expressed in the perisylvian human cortex and apparently determining cerebral asymmetry (Sun *et al.*, 2005; Li *et al.*, 2013); *PCDH11X/Y* (*protocadherin 11 X-linked* and *protocadherin 11 Y-linked*), a gene pair, expressed in the developing human brain and interacting with molecular pathways, implicated in schizophrenia and body asymmetry, with a proposed role in determining brain asymmetry, schizophrenia, and different sex-specific clinical pictures (Priddle and Crow, 2013); and *SRY* (*sex-determining region Y*), apparently determining a different pattern of activity in dopaminergic neurons in the male brain and a different brain morphology in general in men, in comparison with women (Czech *et al.*, 2012). For more details, see a literature review of the five candidate genes in Supplemental digital content 2 (*http://links.lww.com/PG/A94*).

Genomic DNA was extracted from peripheral blood leukocytes using the FlexiGene DNA Kit (Qiagen, Hilden, Germany) following procedures recommended by the manufacturer. For each gene, we selected one or two transcripts (entire cDNA); thus, they contain all known exons in the hg18 human genome assembly. This means that 5′-UTR and 3′UTR were included in the screening. At least 45 base pairs (bp) of intronic sequence bordering each exon were included in the amplicons.

These transcripts were used to design primers with ExonPrimer script from Helmholtz Zentrum München (*http://www.helmholtz-muenchen.de*). The transcripts' names in the UCSC genome browser (*http://genome.ucsc.edu*), their boundaries within the hg18 human genome assembly, the number of amplicons, and the length of amplicons

Table 1 Clinical data on 95 patients, included in the study, classified according to their clinical forms

Clinical form	Mean age of onset	Mean BPRS-24 score	Family history	Sporadic	Continuous course	Episodic course	Men	Women	Total
Paranoid	25.5	58.8	56.6 (43)	43.4 (33)	61.8 (47)	38.2 (29)	55.3 (42)	44.7 (34)	100.0 (76)
Simple	24.7	60.6	55.6 (5)	44.4 (4)	100.0 (9)	0.0	44.4 (4)	55.6 (5)	100.0 (9)
Schizotypal disorder	18.1	62.2	50.0 (4)	50.0 (4)	100.0 (8)	0.0	50.0 (4)	50.0 (4)	100.0 (8)
Hebephrenic	14.5	59.5	100.0 (2)	0.0	100.0 (2)	0.0	100.0 (2)	0.0	100.0 (2)
Altogether	24.6	59.3	56.8 (54)	43.2 (41)	69.5 (66)	30.5 (29)	54.7 (52)	45.3 (43)	100.0 (95)

For family history, course of illness, and sex, percentages are indicated; the actual numbers of patients are in parentheses.
BPRS, Brief Psychiatric Rating Scale.

(the range from smallest to largest values) are indicated for each gene.

LRRTM1: uc002soj.2, chr.2:80367992-80385998; uc002sok.1, chr.2:80381513-80385998; eight amplicons, 388–586 bp.

FOXP2: uc003vgt.1, chr.7:113512617-114059996; uc003vgz.2, chr.7:113841287-114119328; 24 amplicons, 246–616 bp.

LMO4: uc001dmi.1, chr.1:87565738-87584851; uc001dmj.1, chr.1:87568938-87584851; seven amplicons, 283–695 bp.

PCDH11X: uc004efh.1, chr.X:90919959-91026662; uc004efk.1, chr.X:90975314-91765882; 26 amplicons, 248–893 bp.

PCDH11Y: uc004fqm.1, chr.Y:4927266-5034485; uc004fqo.1, chr.Y:4983130-5671264; 26 amplicons, 249–904 bp.

SRY: uc004fqg.1, chr.Y:2713895-2716792; two amplicons, 593–615 bp.

The DNA fragments were amplified by PCR using the C1000 Thermal Cycler (BioRad, Hercules, California, USA) and two DNA polymerases: DNA Taq Polymerase (Fermentas, Vilnius, Lithuania) and Hot Start Taq DNA Polymerase (Sibenzyme, Novosibirsk, Russia). The specificity and yield of PCR products were verified by electrophoresis in a 1.5% agarose gel, stained with ethidium bromide.

Automated Sanger sequencing was performed at two biotechnology companies, Syntol (Moscow, Russia) and Beagle (Saint Petersburg, Russia), using ABI PRISM 3130xl and ABI PRISM 3500 (Applied Biosystems, Carlsbad, California, USA) genetic analyzers, respectively, with Big Dye terminator. The sequencing files were aligned and read using ChromasPro version 1.5 software (Technelysium, South Brisbane, Queensland, Australia).

Given the high degree of identity between *PCDH11X* and *PCDH11Y* genes – more than 98% (Blanco *et al.*, 2000; Ross *et al.*, 2005) – attaining robust specificity for every fragment amplification by PCR, for every male DNA sample, turned out to be problematic as in male DNA samples we could still see a mix of the two genes when they were sequenced. Finding significantly different primer pairs for every PCR fragment of the two genes was not possible. We then decided to include in our study only 43 female DNA samples with respect to the gene *PCDH11X*.

Tools for bioinformatic analysis

To exclude a presence of novel detected variants in the general population, 1000 Genomes release 13 (*http://browser.1000genomes.org/index.html*) and dbSNP 137 (*http://www.ncbi.nlm.nih.gov/snp*) were used. The 1000 Genomes database contains only variants of 1% frequency or more (*http://www.1000genomes.org/about*), whereas dbSNP 137, besides more frequent variants, also contains rare variants of less than 1% frequency (*http://www.ncbi.nlm.nih.gov/projects/SNP/snp_summary.cgi*).

The NCBI Nucleotide database (*http://www.ncbi.nlm.nih.gov/nuccore/*) was used to extract the largest mRNA transcript sizes of the studied genes (which correspond approximately to the exons we screened).

Known microRNA (miRNA)-binding sites within the 3′-UTRs were detected by two prediction programs: TargetScanHuman version 5.1 (*http://www.targetscan.org/*), in which miRNAs of at least 7 bp seeds and conserved across mammals were used, and MicroSNiPer from the Clinical Brain Disorders Branch (*http://cbdb.nimh.nih.gov/microsniper/index.php*), in which human miRNAs of at least 7 bp seeds, listed in miRBase release 19 (*http://www.mirbase.org/*), were used.

Results

Sequencing results

Sequencing results are presented in Table 2. Novel coding variants were not detected. We detected 17 novel variants in total, all of which were found in either 5′-UTR or 3′-UTR of the genes. The variants were not listed in 1000 Genomes release 13 or dbSNP 137. All of the variants were heterozygous single base pair substitutions, except for one single base pair insertion; most of the variants were rare, found in only one individual. The majority of novel variants were found in the genes *LMO4* and *PCDH11X*; no novel variants were found in the *SRY* gene (the 52 male DNA samples were screened). The total number of individuals with the variants was 20; three of the individuals presented two different variants (i.e. two different variants in each individual): sample numbers 17 and 129 within the gene *LMO4* (the two variants in each person could represent a haplotype) and the sample number 58 within the genes *LMO4* and *FOXP2*.

Table 2 Genetic variants found in 95 schizophrenic patients

Gene	Chromosome	Chromosome position (hg19 assembly)	DNA strand	Variant	Variant's context sequence	Position within the gene	Number of chromosome with the variant	DNA samples with the variant	New predicted miRNA-binding sites
LMO4	1	87 794 493	+	G>A	CGGAGGAGGAGGAGGAGCGG(A)AGGAGCGCGGCGGGGGGCGG	5′-UTR	6	17, 22, 58, 68, 130, 156	N/A
LMO4	1	87 794 526	+	G>A	GGGGGCGGGGGCCCCCCGGGC(A)GGGGAATATACAAAGTGAAG	5′-UTR	1	17	N/A
LMO4	1	87 794 531	+	A>C	CGGGGGCCCCCGGGCGGGGG(C)ATATACAAAGTGAAGCCACA	5′-UTR	1	128	N/A
LMO4	1	87 794 897	+	C>T	GCATTTCACCGCCGCCGCCT(T)TCGCAATATTGCAATATAGG	5′-UTR	1	14	N/A
LMO4	1	87 797 403	+	C>A	CGCGCAGCGGAGCCTGCTCT(A)GGAGTTTTGACAGTACCGGA	5′-UTR	1	129	N/A
LMO4	1	87 797 457	+	G>A	GCGGCGGCAAGCGCAGGAAA(A)TTGAGAGGAGCTCGTGGCCC	5′-UTR	1	129	N/A
LRRTM1	2	80 531 435	–	G>C	CCGGCCGCCCTTGCAGCCCC(C)AGCCGAGCAGCAAAGTGAGA	5′-UTR	1	134	N/A
FOXP2	7	114 055 133	+	C>T	ATTTTGTGTACGATTGTCCA(T)GGACGCCAAAACAATCACAG	5′-UTR	2	58, 136	N/A
FOXP2	7	114 330 878	+	T>C	TTTGGTCAGCCTTCTGTAAC(C)TCAATTAGTACAAAGGAACC	3′-UTR	1	11	miR-409; miR-1294
PCDH11X	X	91 034 359	+	C>G	GCCATTCAAAGGGCTGGCT(G)GGCACTGGCTCCTTGCAGTC	5′-UTR	1	166	N/A
PCDH11X	X	91 138 562	+	T>C	CGCCTGTAATCCCAGCACTT(C)GGGAGGCCGAGGCGGGTGGA	3′-UTR	1	151	miR-373; miR-3616
PCDH11X	X	91 138 660	+	C>T	TAAAAATACAAAAAATTAGC(T)TGGCGTGGTGGCGGGCGCCT	3′-UTR	1	24	None
PCDH11X	X	91 876 737	+	A>T	AGTGGCTATTGTGATGTATG(T)TGTATGTAGTCCTACAACTG	3′-UTR	1	16	let-7b
PCDH11X	X	91 877 262	+	A>G	TTATCACACATGGTCATTAA(G)TGGGAAAAAAATAGACTAAA	3′-UTR	1	157	miR-3156-1
PCDH11X	X	91 877 403_91 877 404	+	ins T	CATGGCTATTTCAACATTAG(T)TTTTTTTTGTTTGTTTCTTT	3′-UTR	1	15	N/A
PCDH11X	X	91 877 924	+	C>A	TTTAGTGATTGTAAGTGATA(A)TTTTAAAGAGTAAACTGTG	3′-UTR	1	35	miR-944
PCDH11X	X	91 878 017	+	A>C	TTTATGTTTGTATTGTGGGA(C)GATTCCTCCTCTGTGATATC	3′-UTR	1	146	miR-766; miR-5588; miR-3074

miRNA, microRNA; N/A, not applicable; UTR, untranslated region.

Frequency of novel variations in the genes

From our results, genes *LMO4* and *PCDH11X* appear to be more prone to new genetic variation (six and eight novel variations found, respectively), whereas *LRRTM1* and *FOXP2* appear to be more protected from new genetic variation (one and two novel variations found, respectively). The largest mRNA transcript sizes of these genes (that correspond approximately to the exons we screened), according to the NCBI Nucleotide database (*http://www.ncbi.nlm.nih.gov/nuccore/*), are *LMO4* – 5415 bp; *PCDH11X* – 9190 bp; *LRRTM1* – 3015 bp; and *FOXP2* – 6448 bp. The frequency of novel variations can then be approximated for each gene: one novel variant/kb in *LMO4* and *PCDH11X*, and one novel variant/3 kb in *LRRTM1* and *FOXP2*. As to *SRY*, its largest mRNA transcript size is 897 bp. Novel variations were not detected probably because of the small size of the gene and the fact that only one-half of the cohort (male patients) was used for screening.

Prediction of novel miRNA-binding sites created by mutations

As seven of the single base pair substitutions were located in the 3′-UTRs of the genes (*FOXP2* and *PCDH11X*), we used programs that look for the known miRNA-binding sites within the 3′-UTRs. The results from one program, TargetScanHuman version 5.1, did not show any miRNA-binding sites that included locations of the seven novel variants.

The results from another program, MicroSNiPer, showed multiple binding sites for miRNAs that included locations of the seven novel variants. Moreover, for almost all the variants, except one (C > T, position 91138660 on chromosome X, *PCDH11X* gene), the prediction program indicated that new binding sites for some miRNAs will be created by the variants. These 10 miRNAs are listed in Table 2.

Excluding known mutations in *FOXP2*

We also checked for the presence of two known mutations in the *FOXP2* gene, reported in cases of Speech-language disorder 1 (Lai *et al.*, 2001). These mutations, C > T, leading to R328X, at position 114282671 on chromosome 7, and G > A, leading to R553H at position 114302130 on chromosome 7, were not detected in our cohort of 95 schizophrenic patients.

Discussion

This is the first study in which multiple (five) candidate genes for cerebral LR asymmetry and schizophrenia are analyzed by sequencing, and in particular, using a cohort of 95 schizophrenia and schizotypal disorder patients from Saint Petersburg, Russia. We have not detected any novel coding variants. However, the study allowed to detect 17 novel variants in the untranslated regions

of mRNAs of the genes, not reported in the 1000 Genomes database or dbSNP 137.

Ethnic Russians, constituting almost 81% of the country's population (according to the 2010 All-Russia population census from the Federal State Statistic Service of the Russian Federation), are not genetically separate from other European populations, mainly on the basis of mitochondrial DNA data (Orekhov *et al.*, 1999; Malyarchuk and Derenko, 2001; Malyarchuk *et al.*, 2002; Belyaeva *et al.*, 2003; Malyarchuk *et al.*, 2004). This allowed us to use, in particular, European cohorts from the 1000 Genomes database (500 individuals; *http://www.1000genomes.org/about#ProjectSamples*) as a control population. Saint Petersburg is the second largest metropolis in the Russian Federation and was the capital of Russia for centuries; thus, its population is not isolated. Historically, workers from other parts of Russia came to Saint Petersburg to later constitute its population. It is therefore reasonable to conclude that the population of Saint Petersburg represents the whole of the Russian population and is not different from the 500 individuals constituting European cohorts from the 1000 Genomes database and dbSNP 137.

As we can see from the Results section, *LRRTM1* and *FOXP2* seem to be three times less prone to novel genetic variation, in comparison with *LMO4* and *PCDH11X*. This could be because of the fact that the first two genes have a function, more fundamental for survival, and are more protected from deleterious mutations, whereas the function of the last two genes might be redundant, replaceable by other genes. It is therefore reasonable to expect that a mutation in *LRRTM1* or *FOXP2* will have a greater impact in pathogenesis than a mutation in *LMO4* or *PCDH11X*.

The different results, generated by TargetScanHuman and MicroSNiPer, are expected as the programs rely on different bioinformatic approaches. The use of TargetScanHuman version 5.1, which uses miRNAs conserved across mammals, is justified even if schizophrenia is not a common phenotype in nonhuman animals. The reason is as follows: it seems unlikely that genes, important for brain asymmetry and schizophrenia in humans, are not regulated by miRNAs shared in evolution by different species. Even considering 'human-specific' schizophrenia genes, with a particular role in the cell, these genes still evolved from their homologs in lower animals and should still be regulated by the same molecular machinery in the cell, including the miRNAs. Moreover, given the importance of brain asymmetry in nonhuman animals and the fact that our candidate genes seem to play a role in LR asymmetry of the brain, it is more likely that lower animals and humans share the same miRNAs.

To prove an impact of the found variants on mRNA stability, studies, additional to using the miRNA-binding site prediction programs, are necessary, such as creating cell models in which the integrity of mRNAs with the variants will be measured. Genetic variants in the 3′-UTR part could modify the affinity of miRNAs, regulating the mRNA, for their binding sites, leading to a decrease or an increase in miRNA-regulated mRNA decay (Ul Hussain, 2012). In addition, variants in the 5′-UTR could theoretically modify the process of capping by the capping enzyme or the process of decapping by Dcp proteins (Gu and Lima, 2005). This could lead to a modified rate of the decay of mRNA by exoribonucleases. Altered mRNA amounts in developing neurons in the brain could lead to an incorrect profile of LR brain asymmetry established, later resulting in schizophrenia.

The absence of coding mutations, clearly disrupting the protein structure, in our results is in accordance with the fact that schizophrenia is a polygenic, genetically complex disorder, in which many genes likely contribute toward the clinical picture in a significant proportion of affected individuals (Wray and Visscher, 2010). We should therefore expect finding, in schizophrenia patients, mutations that will not have a detrimental effect on one single protein, as we see in case of Mendelian diseases. Rather, the mutations leading to schizophrenia, although still being absent in the general population, might be in numerous genes in a large proportion of patients and of smaller effect. Recent data on the higher than expected de-novo mutation rate in schizophrenia patients [reviewed in Rees *et al.* (2012)] support the assumption of complex genetics and of high genetic heterogeneity of schizophrenia as de-novo mutations should occur in a large proportion of genes, and not in a few particular genes. In this respect, schizophrenic patients seem to be individuals with a higher mutation load in their genomes than the general population. The genetic heterogeneity of schizophrenia is also in accordance with the fact that the clinical picture of schizophrenia shows a significant variation from one patient to another as well as during the life course of an individual (Boshes *et al.*, 2012). It is hard to explain this variability other than by a simultaneous contribution of many different genes that will vary in different patients.

Therefore, we cannot exclude the genes *LRRTM1*, *FOXP2*, *LMO4*, and *PCDH11X* as candidate genes for schizophrenia because the noncoding mutations found are not present in the general population, including 500 individuals of European origin (1000 Genomes project and dbSNP), and possibly modify the mRNA regulation by miRNAs. As to *SRY*, this gene, to date, does not seem to contribute toward the pathogenesis of schizophrenia.

New and not fully explored, the approach to study the genetics of schizophrenia from the perspective of an LR cerebral asymmetry disturbance deserves more attention. In this study, we analyzed five genes, as described previously (Crow *et al.*, 2009), except for *SRY*. Our results necessitate a follow-up in the form of further genetic screening in different populations and in the form of

functional studies. Moreover, the list of the candidate genes, possibly implicated in LR cerebral asymmetry and schizophrenia, is not limited by the five genes: recently, a new schizophrenia-asymmetry gene was found by association: *CCKAR* (*Cholecystokinin A Receptor*) (Ocklenburg *et al.*, 2013a).

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Conflicts of interest

There are no conflicts of interest.

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Table, Supplemental Digital Content 1

Detailed clinical data for the patients, used in the study.

DNA Sample ID	Gender	Family History	Age of Onset	Age at Examination	Clinical Form	Course of Illness	BPRS-24 score
2	F	N	20	34	1	2	48
3	F	Y	31	56	1	1	58
5	F	N	38	50	1	1	51
6	M	Y	32	42	1	1	55
9	M	N	30	50	1	1	74
11	M	Y	37	54	1	2	71
13	M	N	30	38	1	2	58
14	M	N	19	39	1	1	70
15	F	Y	13	48	1	1	57
16	F	N	33	45	1	1	68
17	M	Y	30	50	1	1	63
18	F	N	47	49	1	1	51
19	M	Y	21	37	1	2	62
20	F	N	35	37	1	2	56
22	M	Y	14	23	4	1	56
23	M	Y	15	30	1	1	63
24	F	Y	44	56	2	1	65
25	M	Y	22	34	1	1	82
26	M	N	22	50	1	1	62
27	M	N	24	58	1	1	81
28	M	Y	20	24	1	1	54
29	M	Y	34	35	1	1	72
30	M	Y	26	63	1	1	63
31	F	Y	21	41	1	1	51
32	F	N	43	48	1	1	57
33	F	N	34	35	1	2	45
34	M	Y	18	62	1	1	57
35	F	N	21	28	1	2	73
36	M	Y	43	69	1	1	54
37	F	Y	24	31	1	2	58
38	M	N	22	25	3	1	63
39	F	N	16	24	3	1	53
42	M	N	25	41	3	1	72
45	F	Y	24	44	1	1	50
49	M	N	23	41	1	2	68
54	F	N	41	51	1	1	72
58	F	N	43	48	1	2	55
59	M	Y	37	43	1	1	63
61	F	Y	23	46	1	1	53
62	F	Y	21	40	1	2	54
65	F	N	18	49	1	2	43
68	M	Y	20	36	1	1	62
71	F	Y	18	22	1	2	54
82	M	Y	20	30	2	1	41
112	M	N	20	24	2	1	68
113	F	Y	15	24	2	1	59
114	M	N	17	41	2	1	69
115	M	Y	18	21	2	1	65

Supplementary Table (continued)

DNA Sample ID	Gender	Family History	Age of Onset	Age at Examination	Clinical Form	Course of Illness	BPRS-24 score
116	M	Y	22	47	1	2	50
117	F	N	29	53	1	1	50
118	M	Y	28	30	1	1	66
119	M	N	21	24	1	1	59
120	M	N	21	31	1	1	65
121	F	N	14	25	1	2	58
122	M	Y	11	23	3	1	57
123	F	Y	14	49	1	2	58
124	F	Y	18	44	1	2	52
125	M	Y	19	54	1	2	58
126	M	Y	30	40	1	1	46
127	M	Y	24	27	1	1	43
128	M	Y	32	36	1	2	43
129	F	Y	38	46	1	1	49
130	M	Y	27	34	1	1	48
131	M	Y	17	26	1	1	83
132	M	N	14	39	1	1	53
133	M	Y	26	55	1	1	75
134	M	N	20	47	1	1	50
135	F	N	22	37	2	1	60
136	F	N	35	46	1	2	50
137	F	Y	13	23	1	1	58
138	M	N	18	32	1	2	53
139	M	N	22	42	1	1	49
141	F	N	22	43	1	2	52
143	F	Y	14	36	3	1	65
145	M	Y	15	26	4	1	63
146	F	N	44	50	1	2	55
148	F	Y	14	43	1	2	62
149	M	Y	22	24	1	2	72
151	F	N	24	28	1	1	71
152	M	Y	44	36	1	1	74
153	F	Y	23	29	2	1	59
154	M	N	20	57	1	2	50
155	M	Y	25	31	1	2	55
156	F	Y	21	47	1	2	40
157	F	N	43	53	2	1	59
158	M	Y	18	33	1	2	72
159	F	N	17	40	1	1	57
160	M	N	21	57	1	1	76
161	M	Y	11	34	3	1	59
163	F	Y	14	32	1	1	76
164	M	Y	14	25	1	1	41
165	F	Y	18	20	3	1	62
166	F	N	28	45	3	1	70
167	F	N	28	44	1	1	61
168	M	Y	36	49	1	1	49

Clinical Form: 1 – Paranoid, 2 – Simple, 3 – Schizotypal Disorder, 4 – Hebephrenic. Course of Illness: 1 – Continuous, 2 – Episodic. BPRS-24 – 24-item Brief Psychiatric Rating Scale.

Literature review of the candidate genes, Supplemental Digital Content 2

LRRTM1 (Leucine Rich Repeat Transmembrane Neuronal 1)

The gene region was first discovered by linkage (Francks, Fisher *et al.* 2002, Francks, DeLisi *et al.* 2003a, Francks, DeLisi *et al.* 2003b) and later the gene was associated with schizophrenia and handedness (Francks, Maegawa *et al.* 2007). Schizophrenia patients are statistically more frequently non-right-handed (Sommer, Ramsey *et al.* 2001) which points to a possible link between handedness and schizophrenia. Handedness is reflected in brain structural or functional LR asymmetry, the central sulcus is being regarded as a potential anatomical correlate of hand preference (Renteria 2012). Therefore, the genes, responsible for the asymmetric development of brain areas that govern the hand use, might also be important for LR asymmetry, in other brain structures, that is affected in case of schizophrenia. LRRTM1 is believed to be one of such genes. Its specific role in brain function is still not completely clear, but the protein product seems to be important for maintaining synaptic integrity; mice with this gene being knocked out show significant cognitive impairment (Linhoff, Lauren *et al.* 2009, Takashima, Odaka *et al.* 2011).

FOXP2 (Forkhead Box P2)

This is a transcription factor, expressed in the human brain, mutated in case of Speech-language disorder 1 (Lai, Fisher *et al.* 2001). Two SNP in this gene are associated with hemispheric asymmetries for speech perception (Ocklenburg, Arning *et al.* 2013). Since language areas of the human brain are asymmetric on the LR axis, and since structural and functional LR asymmetry is compromised in case of schizophrenia, language and schizophrenia might have common mechanisms.

There is also additional opinion among many authors that language, cerebral asymmetry and schizophrenia are specific only to humans, appeared with the emergence of *Homo sapiens* and therefore they could all depend on a common genetic factor (reviewed in (Crow 2008)). This point of view – one language-schizophrenia gene present only in humans – is rather debatable, since chimpanzees and bonobos also use various ways of

language-like communication (Corballis 2002, Pollick, de Waal 2007), while language cortical areas are present and are asymmetric in all great apes (Cantalupo, Hopkins 2001, Spocter, Hopkins *et al.* 2010) (some authors contest these findings (Schenker, Hopkins *et al.* 2010, Chance, Sawyer *et al.* 2012)). Moreover, even frogs possess a vocal generator localized asymmetrically in the left brain hemisphere (Bauer 1993), suggesting early evolution of common prerequisites for the vocal communication in all vertebrates. The role of FOXP2 in vocal production and learning has strong parallels between humans and birds: the reduced level of expression of FOXP2 in basal ganglia by RNAi leads to inaccurate vocal imitation in zebra finches (Haesler, Rochefort *et al.* 2007). Also, FOXP2 expression in the developing brain of mouse and human was found to be strikingly similar (Lai, Gerrelli *et al.* 2003). Hence, cerebral lateralization and vocal communication in lower animals, although not as evolved as in humans, are not specific only to humans. Moreover, researchers have managed to create rodent models with elements of schizophrenia (Powell, Miyakawa 2006). If the schizophrenia gene (or genes) has some role in cerebral asymmetry and language, then the gene is present not only in humans.

The previous genetic studies of FOXP2 in schizophrenia patients were somewhat contradictory (Sanjuan, Tolosa *et al.* 2005, Sanjuan, Tolosa *et al.* 2006, Tolosa, Sanjuan *et al.* 2010, Spaniel, Horacek *et al.* 2011, Li, Zeng *et al.* 2012), not always reporting significant association between schizophrenia and SNPs in FOXP2. On the other hand, DISC1 (Disrupted in schizophrenia 1) gene, one of the best candidates for genes, playing a role in schizophrenia, was found to be inhibited by the transcription factor FOXP2 (Walker, Hill *et al.* 2012), which traces another link between FOXP2 and schizophrenia. Also, FOXP2 appears to regulate neuronal maturation and migration during brain development in mice, although the results of different studies indicate either negative (Clovis, Enard *et al.* 2012), or positive (Vernes, Oliver *et al.* 2011) regulation of neuronal development. Finally, FOXP2 was found to be expressed in the cortical plate, basal ganglia, thalamus, inferior olives and cerebellum in the developing brain of mouse and human. These findings support a role for FOXP2 in the development of corticostriatal and olivocerebellar circuits involved in motor control, and this in turn can be related to the speech (articulation) deficits, and probably also to linguistic and grammatical (cognitive) impairments, observed in Speech-language disorder 1 (Lai, Gerrelli *et al.* 2003). Incorrect development of brain areas, involved in some aspects of the language faculty, could have consequences on development of

asymmetric areas of the brain. This can make FOXP2 a candidate of choice for a gene, important in LR asymmetry of the human brain and in schizophrenia.

LMO4 (Lim Domain Only 4)

LMO4 is required for neural tube closure, neural crest formation, as well as for maturation and migration of neurons in the developing brain cortex in mice and frogs (Lee, Jurata *et al.* 2005, Asprer, Lee *et al.* 2011, Ochoa, Salvador *et al.* 2012). Additionally, this transcription co-factor was found to be consistently asymmetrically expressed in the perisylvian human cortex (Sun, Patoine *et al.* 2005) that includes Wernicke's language comprehension area, one of the most clearly asymmetric areas of the human brain (Renteria 2012). This expression asymmetry – higher expression on the right side – was found only in 12-, 14-, and 17-week old, but not in 19-week old human embryonic brains, so LMO4 could be one of the genes, involved in development of LR asymmetry of the human brain cortex. Moreover, LMO4 knockout or suppression in mice points to a role of LMO4 in formation of cortical areas, important for sensory perception and motor coordination (Kashani, Qiu *et al.* 2006, Huang, Kawase-Koga *et al.* 2009), and LMO4 knockdown only on the right side in embryonic cortices in mice, that normally do not exhibit brain functional asymmetry, results in the right paw preference, as well as suppression of early neurogenesis in the right hemisphere (Li, Bian *et al.* 2013). Again, if this gene's function is compromised, it could lead to abnormalities in LR asymmetry of the brain and therefore to schizophrenia.

PCDH11X/Y (protocadherin 11 X-linked and protocadherin 11 Y-linked gene pair)

The gene pair is found in the region of homology between the human sex chromosomes (Xq21.3/Yp11.2), is expressed predominantly in the brain – in the adult, as well as in the embryo – and seems to take part in the brain circuit formation and maintenance by determining the cell-cell recognition (Blanco, Sargent *et al.* 2000, Kim, Yasuda *et al.* 2011, Priddle, Crow 2012). A role in schizophrenia for this gene pair was proposed by the

British psychiatrist Timothy J. Crow, who also proposed the notion that schizophrenia is a genetic disorder of altered LR asymmetry of the brain (Crow, Ball *et al.* 1989, Crow 2002, Crow 2008). The argument of T.J. Crow is the following: schizophrenia is a human-specific illness; language is a human-specific faculty; in schizophrenia, LR asymmetry of the cortical structures is reduced or reversed; language ability is determined by the highly asymmetric brain structures in humans; schizophrenia therefore could be the consequence of humans acquiring the faculty of language; schizophrenia is an illness, arising from altered LR asymmetry of the brain; LR brain asymmetry profiles in men and women are different; in men, the age of onset of schizophrenia is earlier and the incidence of the illness is more frequent (Boshes, Manschreck *et al.* 2012, Cascio, Cella *et al.* 2012); sex-chromosome aneuploidies are characterized by verbal and spatial deficits, related to LR brain asymmetry alteration; the genetic factor, underlying schizophrenia, language, and LR brain asymmetry has to be unique to humans and has to be on sex chromosomes, but in a region of similarity between the two sex chromosomes (so there would be two similar, but not identical versions of the gene). The only such genetic candidate is the PCDH11X/Y gene pair: because it appeared as a result of duplication from X chromosome at the time of the chimpanzee and hominid separation, it is now present only in humans (Williams, Close *et al.* 2006). Each gene in the pair has numerous (5 to 16) changes in the coding sequence, leading to amino acid substitutions, in comparison with the sequence of PCDH11X in the great apes. Because the gene pair is expressed in the brain, male and female neurons have a different gene content: men have PCDH11X and PCDH11Y and women have 2 copies of PCDH11X, since the gene escapes X-chromosome inactivation (Lopes, Ross *et al.* 2006). Both genes are highly expressed in the developing cerebral cortex: in fetal (12–34 weeks postconception) and adult human brains PCDH11X/Y expression was detected in developing neurons as they migrated from the ventricular zone, through the subplate and into the cortical plate; both proteins interact with β-catenin, a protein that plays a role in determining axis formation and regulating cortical size (reviewed in (Priddle, Crow 2013)).

WNT pathway, which includes β-catenin and GSK3β (glycogen synthase kinase 3 beta), was found to be directly implicated in schizophrenia (reviewed in (Lovestone, Killick *et al.* 2007, Emamian 2012)) and in cerebral asymmetry: WNT pathway determines habenula asymmetry in zebrafish (Husken, Carl 2012). In

particular, atypical and typical antipsychotic drugs alter GSK3 (GSK3α and β) activity, as do drugs that induce psychosis; many of the genes statistically associated with schizophrenia directly or indirectly regulate GSK3 activity (Lovestone, Killick *et al.* 2007). GSK3β is also statistically associated with schizophrenia and other psychiatric disorders (Benedetti, Bernasconi *et al.* 2004, Li, Mo *et al.* 2011). This indicates a possible role of PCDH11X/Y gene pair in determining cerebral asymmetry and playing a role in pathogenesis of schizophrenia.

Genetic studies of this gene pair in schizophrenia patients were not numerous and did not show an involvement of the gene pair in schizophrenia. In particular, T.J. Crow and colleagues (Giouzeli, Williams *et al.* 2004) did not find disease-causing mutations in the gene pair in 214 affected individuals, when compared to their unaffected relatives from 99 families, using the DHPLC. Also, an association study of two non-synonymous SNPs in the PCDH11Y gene did not show any association with schizophrenia (Durand, Kappeler *et al.* 2006). Nevertheless, the gene pair deserves attention when studying the genetics of schizophrenia in the light of altered LR asymmetry of the brain.

SRY (sex determining region Y)

This is a transcription factor, found on Y chromosome, which participates in the testis determination (Marshall Graves 2002, Wilhelm, Palmer *et al.* 2007). Known mutations in this gene lead to gonadal dysgenesis (Filges, Kunz *et al.* 2011). Additionally, the gene evolutionally derives from the X chromosome-linked SOX3 (SRY-box 3) gene (Marshall Graves 2002), part of the SOX gene family, responsible, among other functions, for the nervous system development. SRY might therefore confer different properties to male neurons, in comparison with female neurons, expressing only SOX3 (Turner, Ely *et al.* 2011). In particular, SRY may play a role as a positive regulator of dopamine synthesis and metabolism in the human male midbrain, being expressed in a sub-population of tyrosine hydroxylase-positive neurons in the substantia nigra pars compacta and in the ventral tegmental area (Czech, Lee *et al.* 2012). Also, SRY was found to be expressed in the rodent substantia nigra, in dopaminergic neurons, and regulates tyrosine hydroxylase expression (Dewing, Chiang *et al.* 2006). Therefore, SRY could determine a different pattern of activity in dopaminergic neurons in the male brain and different

brain morphology in general in men, in comparison with women. This can be linked to the fact that schizophrenia has a different incidence in male and female patients (Boshes, Manschreck *et al.* 2012, Cascio, Cella *et al.* 2012): SRY could play a role in a "male-specific" schizophrenia phenotype (in a way, similar to PCDH11Y), which tends to have an earlier age of onset and a greater incidence of the illness. The SRY gene, apart from possibly determining the different brain morphology in men, could also determine the male-specific profile of LR brain asymmetry. In addition, the involvement of the dopaminergic system in schizophrenia is well characterized (Freedman 2003), which adds another possible link between schizophrenia and SRY. Although known mutations lead to gonadal dysgenesis, sex-chromosome aneuploidies are characterized by verbal and spatial deficits, related to LR brain asymmetry abnormalities (reviewed in (Crow 2008)). This indicates that these verbal and spatial deficits, related to LR brain asymmetry abnormalities, could be due to a loss of SRY. In case of this gene, the genetic transmission would be in the males only within a family.

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