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ORIGINAL PAPER

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Family-based study of markers at the 5′-flanking region of the human dopamine transporter gene reveals potential association with schizophrenic psychoses

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Abstract The dopamine hypothesis of schizophrenia proposes an inherited or acquired presynaptic hyperactivity of dopaminergic neurons. The human dopamine transporter gene (hSLC6A3; hDAT) represents one major mechanism for the termination of dopaminergic neurotransmission. This study examines the degree of genetic association of the 5′-untranslated region (5′-UTR) of the hSLC6A3 to schizophrenia in a family-based association design. Five single nucleotide polymorphisms (SNPs) derived by a previous systematic mutation scan ~1.2 kb of the 5′-UTR of the hSLC6A3 locus were genotyped for transmission disequilibrium between 82 index cases (56 males) with schizophrenia and their biological parents. We observed no preferential transmission of alleles from heterozygous parents to affected offspring. Five estimated haplotypes accounted for a frequency of 90% in the index cases, and were identical in cases and non-transmitted parental control haplotypes. Distinct five-locus-genotypes accumulated in schizophrenia compared to parental controls at *P*-value 0.0038 with odds-ratio of 2.02 (95% CI 0.99–4.14). In conclusion, our present findings support the genetic involvement of distinct hSLC6A3 genotypes in schizophrenia. We propose replication in extended samples and examination of the functional relevance of the associated genotypes on human dopamine transporter expression.

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Introduction

Dysbalances of mesolimbic dopaminergic pathways are thought to be one of the basic mechanisms underlying psychotic disorders [1]. Dopamine dysregulation in schizophrenia should be viewed in the context of dopamine biosynthesis and release, the rate of diffusion, reuptake, and degradation. The dopamine transporter-mediated reuptake is the primary mechanism for limiting the extent, duration, and area of dopamine receptor activation. Neuroleptic-naïve first-episode schizophrenic patients show unaltered striatal dopamine transporter density, but lack the asymmetry in the caudate [2] and show an inversely correlation of dopamine transporter availability to the extent of hallucinations [3]. The human dopamine transporter (hSLC6A3; hDAT) is a member of the Na^+- and Cl^-dependent solute transporter family with 623 amino acids, organized as a tetramer in the plasma membrane. SLC6A3 mediates efflux of dopamine from cytoplasmatic to extracellular compartements, and terminates dopaminergic neurotransmission by reaccumulation of dopamine into presynaptic neurons [4, 5]. Human SLC6A3 is located at chromosomal region 5p15.33 at ~1.4 Mb and consists of 15 exons, spanning ~60 kb on genomic DNA [6, OMIM: *126455).

Genetic linkage of schizophrenia to chromosome 5p14-13 is mainly supported from the analysis of individual multiplex pedigrees [7, 8], whereas most genome-wide linkage scans and meta-analytic approaches were not successful in delineating a major gene locus at that region [9–11]. Considering hSLC6A3 as a functional candidate gene, genetic association studies have mostly used a variable number of tandem repeat (VNTR)-polymorphism in the 3′-flanking region of hSLC6A3. However, a preferential transmission

of VNTR alleles was not supported by family-based studies [12, 13, 14], and likewise a meta-analysis of case–control studies excluded distinct VNTR alleles as factors for increased liability to schizophrenia [15]. There exists only one systematic mutation scan of the coding region of hSLC6A3 in index cases with schizophrenia; the detected polymorphisms, however, were not found associated with disease [16]. We had previously performed a systematic mutation analysis of ~1.6 kb of the 5′-untranslated region (5′-UTR) encompassing the entire promoter region and the non-coding exon 1 [17], resulting in delineation of five diallelic polymorphisms. Here, we report the results of a family-based association study of schizophrenic psychoses with these 5′-UTR derived markers.

Individuals and methods

At the human SLC6A3 5′-UTR locus we performed a family-based association study on 82 index cases (56 males) with schizophrenia, which were recruited from the Department of Psychiatry at the University of Würzburg, and their biological parents. A consensus diagnosis of schizophrenic psychoses according to DSM IV/ICD10 criteria was established in all of them. Cases had a mean age at onset (first hospitalization) of 23.0 ± 6.2 years and an age at assessment of 29.8 ± 7.3 years. In 24 triads (29%) we found a parent (7 males) with previous hospitalization for schizophrenia (consensus diagnosis derived by clinical interview and hospital records). There was no evidence for bilineal transmission in the sample. All subjects were unrelated and of German Caucasian descent. The Ethics Committee of the University of Würzburg had approved the study, and informed consent was obtained from all subjects.

Previously, we had performed a systematic mutation study of the 5′-UTR between −1586 basepair (bp) and +97 bp relative to the transcription start site (GenBank D88556; [18]) in 120 individuals (40 cases with schizophrenic disorders, bipolar affective disorder, and 40 healthy controls each). The mutation scan resulted in five diallelic single nucleotide polymorphisms (SNPs) found in each of the diagnostic samples. The numbering is according to the SNP databases at http://snp.cshl.org/ and http://ncbi.nlm.nih.gov/snp. The index cases analyzed in the family-based association study were independent from those in the mutation scan. PCR-based restriction fragment length polymorphism (RFLP) assays were used to assess the frequency of the respective variants (for details: see [17]. Original primer (5′-3′) sets were used for rs2975226 (F: CCAGCCA TCTGCGTCC, R: GATGC CGAGAGCGACG), ntC>G (F: TGGAGCCGGATACCAA CC, R: CTCCACGCTCTGACCAGC), rs2652511 (F: GCTCACGG GAGCATCGAG, R: GCACTCGCCTAAGAAAAC CA), and rs6413429 (F: ACAGCTTCGAGGTGGCAC, R: CTGTGTCT GGTGAGGGCC). In the case of rs2617596, we performed a modified PCR to produce a PCR-product of 143 bp with an artificial restriction site (FM: AG CCCCGGAGCCGGGGCGC, R: GAGGGAGGCAGGGA CCCTTGG). PCR [30 s at 94°C, 30 s at 63°C (fragment 5: 57°C), and 30 s at 72°C for 36 cycles] was carried out in 25 μl volume containing 40 ng genomic DNA, 20 pmol of each primer, 75 mM Tris-HCl (pH 8.8 at 25°C), 20 mM $(NH_4)_2SO_4$, 0.01% (v/v) Tween 20, 1.5 mM Magnesium Chloride, 200 μM of each dNTP, and 0.5 U Taq DNA polymerase (PeqLab, Erlangen, Germany). After amplification of genomic DNA 5 μl of PCR product were digested with Tth111I, HhaI, BstUI, MspI, and DdeI (New England Biolabs). Fragments were resolved on a 10% PAA gel containing 1.0 × TBE at 15 V/cm. Bands were visualized by silverstaining.

The exact test proposed by Weir [19] was applied to test if the distribution of genotypes in control individuals (obtained by combining the two non-transmitted parental alleles) deviates from Hardy–Weinberg equilibrium. The program FAMHAP [20] was used to estimate haplotype frequencies and to calculate the pairwise standardized linkage disequilibrium coefficient D'. The significance of the observed transmission disequilibrium for alleles at a single marker locus and for haplotypes consisting of alleles at two or more SNPs was assessed by the transmission-disequilibrium tests (TDT) [21] and an extension of the TDT proposed by Zhao et al. [22] for multilocus testing of tightly linked markers with a randomitation procedure. Fisher's exact test as implemented in the SAS was used to compare the distribution of five-locus-genotypes between cases and controls. Power calculations are based on the approximation described by Knapp [24]. We conducted a computational analysis of the five diallelic SNPs using PROSCAN Version 1.7 (http://thr.cit.nih.gov/; http://www.gene-regulation.com), a program that predicts promoter regions based on scoring homologies with putative eukaryotic Pol II promoter sequences.

Results

In 82 triads with schizophrenic psychoses, we observed no preferential transmission for any of the marker alleles at the 5′-flanking region of hSLC6A3

Table 1 Transmission of markers at hSLC6A3 in 82 triads with schizophrenic Psychoses

SNP (nt position)	rs2975226 (1498616 T > A)			- (1499209 C >G)			rs2652511 (1499389 C > T)			rs2617596 (1499719 C > G)			rs6413429 (1500027 T > G)		
MT	11	12	22	11	12	22	11	12	22	11	12	22	11	12	22
11 × 11	2	-	-	75	-	-	5	-	-	6	-	-	67	-	-
11 × 12	4	8	-	3	3	-	15	13	-	16	13	-	5	6	-
11 × 22	-	11	-	-	0	-	-	7	-	-	6	-	-	0	-
12 × 12	7	9	7	1	0	0	7	12	8	10	17	6	1	2	1
12 × 22	-	15	13	-	0	0	-	9	2	-	5	1	-	0	0
22 × 22	-	-	6	-	-	0	-	-	4	-	-	2	-	-	0
n%	13 (15.9)	43 (52.4)	26 (31.7)	79 (96.3)	3 (3.7)	0 (0)	27 (32.9)	41 (50.0)	14 (17.1)	32 (39.0)	41 (50.0)	9 (11.0)	73 (89.0)	8 (9.8)	1 (1.2)
P(HWE)	0.50			0.06			0.28			1.00			0.21		
TDT	42 T/44 NT			5 T/3 NT			50 T/43 NT			58 T/43 NT			9 T/10 NT		
P(TDT)	0.83			0.48			0.47			0.14			0.82		

Nucleotide numbering is according to contig NT_006576 (EMBL/GenBank); SNP database: http://snp.cshl.org/; http://ncbi.nlm.nih.gov/snp
MT = mating type; for each SNP the two parental genotypes are given (*y*-axis) and the frequency of the geneotypes transmitted to the offspring (*x*-axis); n (%) gives the genotype distribution for each SNP in the index cases. HWE = Hardy–Weinberg-Equilibrium in parental control alleles; Transmission-Disequilibrium Test (TDT) gives the P-value derived by the number of the heterozygous parents who have transmitted (T) or non-transmitted (NT) alleles 1 to the offspring; *P* = significance level

(Table 1). Subsequent analyses regarding gender of the index case as well as comparisons between paternal or maternal transmission were non-significant (data not shown). The genotype distribution of the SNPs satisfied Hardy–Weinberg equilibrium in the combined two non-transmitted parental alleles. Between rs2975226, rs2652511, and rs2617596, we observed highly significant inter-marker linkage disequilibrium (rs2975226 – rs2652511: $D' = -0.82$; rs2975226 – rs2617596: $D' = -0.69$; rs2652511 – rs2617596: $D' = 0.75$).

Distinct five-locus-genotypes accumulated in the schizophrenic offspring at *P*-value 0.0038 comparing the frequencies of the six most common genotypes and the pooled genotypes with frequencies <5.0% between the index cases and the control individuals (Fisher's exact test; Table 2). The three most common five-locus-genotypes among index cases had a frequency of 64.7% compared to 48.8% in parental controls. Mainly, the 5-locus-genotype of nt 1498616TA – 1499209CC – 1499389CT – 1499719CG – 1500027TT was overrepresented in the schizophrenic offspring with 32.9% compared to 19.5% in parental controls. Odds-ratio for the five-locus-genotypes was 2.02 (95% CI 0.99 – 4.14). The transmission-disequilibrium-test (TDT) with five-loci gave non-significant results ($P = 0.10$), but three-loci and four-loci TDT produced a marginal hint for transmission distortion of $P = 0.024$ for rs2652511, rs2617596, and rs641342 ($T = 14.94$). Estimated five-locus haplotype frequencies were similar in index cases and parental control alleles (Table 3). Maximum-likelihood (ML) estimation of five-locus-haplotypes resulted in an estimated frequency >0 for 12 out of 32 theoretically possible haplotypes with no differences between transmitted and non-transmitted haplotypes ($\chi^2 = 30.71$; df 31; $P = 0.48$). The study sample was relatively small, but our family-based study with 82 triads possesses a power of 80% to detect (at $\alpha = 0.05$) an association with a susceptibility allele, under the assumption that the susceptibility allele has a population frequency of 0.5, and the effect of this allele is recessive with a relative risk of 2.8.

Discussion

At the 5′-flanking region of the human dopamine transporter gene (hSLC6A3), we found distinct 5-locus-genotypes significantly overrepresented at *P*-value 0.0038 in subjects with schizophrenia compared to parental control genotypes. The five-locus-genotype (nt) 1498616TA – 1499209CC – 1499389CT – 1499719CG – 1500027TT was observed at a frequency of 32.9% compared to 19.5% in non-transmitted alleles resulting in an odds ratio of 2.02 (95% CI 0.99 – 4.14). Transmission disequilibrium tests with individual markers showed equal transmission at the hSLC6A3 locus, but TDT with three-loci or four-loci gave a marginal hint for transmission distortion of $P = 0.024$ for rs2652511, rs2617596 and rs641342 ($T = 14.94$). However, this result was challenged by the observation

Table 2 Genotypes at the 5′-UTR of hSCL6A3 in schizophrenic psychoses

rs2975226 (1498616 T > A)	- (1499209 C > G)	rs2652511 (1499389 C > T)	rs2617596 (1499719 C > G)	rs6413429 (1500027 T > G)	Index cases	%	Parental controls	(%)
TA	CC	CT	CG	TT	27	(32.9)	16	(19.5)
AA	CC	CC	CC	TT	18	(22.0)	15	(18.3)
TT	CC	TT	GG	TT	8	(9.8)	9	(11.0)
TA	CC	CC	CC	TT	5	(6.1)	0	(0.0)
TA	CC	CT	CC	TT	2	(2.4)	6	(7.3)
AA	CC	CC	CG	TT	0	(0.0)	7	(8.5)
		Others			22	(26.8)	29	(35.4)

Distinct five-locus-genotype accumulated in schizophrenic disorders compared to the two non-transmitted parental control alleles at p-value 0.0038 (Fisher's exact tests). Odds-ratio for the five-locus-genotype 1498616TA – 1499209CC – 1499389CT – 1499719CG – 1500027TT was 2.02 (95%CT 0.99–4.14).

Table 3 Estimated haplotype frequencies of the five-markers at hSLC6A3

rs2975226 (1498616 T > A)	- (1499209 C > G)	rs2652511 (1499389 C > T)	rs2617596 (1499719 C > G)	rs6413429 (1500027 T > G)	T %	NT %
A	C	C	C	T	46.0	41.0
T	C	T	G	T	31.0	29.0
T	C	T	C	T	7.0	6.0
A	C	C	C	G	5.0	3.0
A	C	C	G	T	1.0	5.0
			Others		10.0	16.0

Transmitted (T) and non-transmitted (NT) haplotypes with a frequency ≥ 3% in the total sample are depicted
Maximum-likelihood (ML) estimation of 5-locus-haplotypes gave no differences between transmitted and non-transmitted haplotypes ($\chi^2 = 30.71$; df = 31; $P = 0.48$).

that estimated 5-locus-haplotype frequencies were similar in cases and parental control alleles.

While lacking a high-risk haplotype at the hSLC6A3 locus, the 5-locus-genotype was the major hint for an involvement of hSLC6A3 in schizophrenic disorders obtained in our study. Thus, dopaminergic dysregulation in a complex trait as schizophrenic psychoses may be linked to a complex, recessively transmitted variation of hSLC6A3 expression. Unfortunately, we could not replicate the association of rs 2975226 to schizophrenia found in a single marker association study of 100 cases and controls, each [26]. Compared to our findings of TT 16%, TA 52%, and AA 32% in cases with schizophrenia, the genotype frequencies were similar in the Iranian sample with TT 12%, AT 59%, and AA 29%, but the genotype distortion in the control sample might be responsible for the statistical association (TT 5%, AT 38%, and AA 57%).

The same five-locus-genotype has been found at an increased frequency of 41.9% compared to 28.1% in a case–control association study on 105 index cases with bipolar affective disorder and 199 controls with odds ratio of 1.84 (95% CI 1.12 – 3.02) [45]. This raises the question, whether alterations of hSLC6A3 expression – as suggested for the serotonin transporter – are related to common phenomena in various neuro-psychiatric disorders. Non-specific symptoms, such as psychomotor agitation or inhibition, affective and cognitive disintegration are shared by various disorders which are related to dopaminergic transmission, e.g. Parkinson's disease, alcoholic delirium, the major psychoses, attention deficit hyperactivity disorder, or amphetamine abuse with psychosis [4, 5, 25, 23]. If the association is replicated in extended samples and different disorders, this may point to small, albeit significant contribution of distinct genotypes at the hSLC6A3 locus to the development of the major psychoses or other neuropsychological syndromes.

Dysregulation of hSLC6A3 expression could be related to a particular combination of polymorphisms across the gene, including the 5′-UTR SNPs. The distal promoter region (at about −2.5 to −7.8 kb) and the first intron spanning ~2.2 kb seem to display further potential enhancer and silencer elements [27], but have not yet systematically screened in patient populations. The 40-bp variable number of tandem repeat (VNTR)-polymorphism at the 3′-UTR of SLC6A3 has attracted much attention, but is mainly excluded to strongly affect hSLC6A3 expression or of being associated with the major psychoses in numerous single SNP-association studies and meta-analyses [13, 15]. However, segmental LD was apparent at the SLC6A3 locus in an analysis of 14 SNPs and the VNTR with suggestive evidence of an association with bipolar affective disorder [28, 29]. Since the 5′-UTR of SLC6A3 was covered only by a cluster of SNPs in intron 1 and two SNP separated by a distance of ~2.5 kb in the 5′-UTR [28, 30], our data could add evidence that genotypes at the 5′-UTR near the core promoter may also be involved. The coding region seems to be relatively conserved in individuals with schizophrenia or other clinical syndromes [16, 31, 32], and subsequent association studies in extended samples were essentially negative. Based on the availability of multiple markers on the 5′- and 3′-UTR, future studies could clarify the exact borders of LD-blocks and could delineate the degree of association to disease.

The biological role of any of these 5′-UTR markers on hSLC6A3 transcriptional regulation solitary or in combination are yet unexplored. They are located within a core region that regulates the activity of luciferase constructs with 10–150 times more activity than promoterless constructs [33]. Particularly, two conserved GC-boxes in the core promoter region around −200 bp to the transcription start site seem to be essential for the transcriptional activity of hSLC6A3 in neuroblastoma cell lines [34]. Although the TATA-like promoter sequence, the two core promoter GC-boxes and a conserved neuron-specific CCAGGAG motif were not found polymorphic; there are numerous other proposed silencing elements in intronic and the distant 5′-UTR, outside the strong proximal core promoter region [27, 35]. Since transcription factor binding site prediction is a difficult task, we newly conducted a computational analysis of the five diallelic SNPs, which should replace our earlier suggestions [17]. The analysis revealed that nt 1499209C > G is located within a putative SP1-binding site [36] and nt 1499209G introduces a LF-A1 binding site [37]. Whereas Sp1-binding sites are ubiquitously expressed in mammalian cells [38], the transcription factor LF-A1 interacts with the promoter regions of several genes expressed in hepatocytes [37, 39]. At rs6413429 the sequence change replaces a wild-type POUF-1 (Pit-1) factor [40], in which mutations have been reported as rare causes of combined pituary hormone deficiency [41, 42]. We found no alteration of a putative transcription binding site at rs2975226, which is at 9 nt distance to the second core-promoter GC-box, and found the sequence (nt 1498616T) not conserved between human and mouse [34].

Decreased striatal dopamine transporter binding was observed in stable, medicated chronic schizophrenic patients using (^{18}F)-CFT-positron-emission tomography [43], while neuroleptic-naïve first-episode schizophrenic patients showed mainly unaltered dopamine transporter binding with right–left asymmetry in distinct brain areas or inversely related to the acute illness phase [2, 3, 44]. On the one hand, this would be in line with the assumption of decreased expression of hSLC6A3 during the disease process or under continuous medication more than a pre-existing of structural and functional deficits of dopaminergic nerve terminals. On the other hand, the

potential genetic effect at the dopamine transporter gene locus is – if any –small and favours a complex genetic interaction in pre- and postsynaptic dopaminergic function or a non-specific contribution to symptoms found in various neuropsychiatric disorders.

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