Evidence for a negative association between schizophrenia and a polymorphism in the insulin receptor substrate-3 (*IRS-3*) gene

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Abstract

OBJECTIVES: Since there are clear indications that schizophrenia is a systemic disorder, we sought for a common molecular basis for schizophrenia abnormalities in brain and body. Our hypothesis was that an impaired insulin and insulin-like growth factor signalling in cells might underlie changes in both brain and body in schizophrenia. In this regard, the insulin receptor substrates 1–4, linking both the insulin and insulin-like growth factor-1 receptors with intracellular pathways, might be of interest to study genetically. In the present study, we chose to study the insulin receptor substrate-3 (*IRS-3*) gene as a candidate gene in schizophrenia.
METHODS: The *IRS-3* gene of 93 patients with the diagnosis of schizophrenia according to DSM-IV criteria and 57 healthy control subjects was screened for DNA sequence variations, followed by case-control analyses of total 10 detected polymorphisms.
RESULTS: The A/G genotype of the single nucleotide polymorphism (SNP) rs117078492 in the *IRS-3* gene occurred in 5.3% of the control subjects compared with in 0% of the patients (p=0.05). Similarly, the haplotypes 5 and 3X, constructed from polymorphisms in the *IRS-3* gene and including the A allele of this A/G SNP, occurred only in the control subjects and not in the patients (5.3% vs 0%, p=0.05).
CONCLUSION: Our findings suggest that individuals carrying the A allele of this A/G SNP in the *IRS-3* gene as well as the estimated haplotypes 5 or 3X including this A allele, have a protection against schizophrenia development.

ORIGINAL ARTICLE

INTRODUCTION

The literature on the schizophrenia illness provides strong evidence for a role of genetic factor(s) in its aetiology (Craddock *et al.* 2005). A variety of genes, each with small or moderate effect, have been suggested to be involved in the aetiology of the disorder (Gottesman & Shields 1967), and in recent years there have been several reports on such genes as those encoding α1C subunit of the L-type voltage-dependent calcium channel (CACNA1C), catechol-O-methyltransferase (COMT), disrupted in schizophrenia 1 (DISC1), dysbindin (DTNBP1), G72, golli-myelin-basic-protein (MBP), neuregulin 1 (NRG1), neurexin 1 (NRXN1), serotonin receptor 2A (HTR2A), zinc finger 804A (ZNF804A) and others (Baruch *et al.* 2009; Chumakov *et al.* 2002; Craddock *et al.* 2005; Egan *et al.* 2001; Green *et al.* 2009; Melkersson & Hulting 2009; Nyegaard *et al.* 2010; O´Donovan *et al.* 2008; Ptacek *et al.* 2011; Ripke *et al.* 2011; Rujescu *et al.* 2009; Stefansson *et al.* 2009, 2002; Straub *et al.* 2002). However, the main genetic factor(s) associated with schizophrenia still remain(s) to be found.

There are also clear indications that schizophrenia is a systemic disorder and not only a brain disease (Flyckt 2001). Therefore, we sought for a common molecular basis for schizophrenia abnormalities in brain and body, and found an interesting hypothesis, described more in detail in two recent studies (Melkersson & Persson 2011; Melkersson *et al.* 2011) that impaired cellular signalling via the insulin receptor (IR) and possibly also via the insulin-like growth factor-1 receptor (IGF-1R) might underlie known abnormalities associated with schizophrenia in both the central nervous system (CNS) (i.e. structural and functional changes) and in peripheral organ systems (i.e. growth dysregulation, impaired glucose tolerance, lowered resting energy expenditure and neuromuscular dysfunction).

The IR and IGF-1R are both present in CNS and peripheral organs in humans (McCowen & Smith 2005; Rui & White 2004; Sara *et al.* 1982). However, there are no reports on the *IR* gene (located on chromosome 19p13.3–p13.2) and schizophrenia, and between single nucleotide polymorphisms (SNPs) in the *IGF-1* or *IGF-1R* genes (located on chromosomes 12q23.2 and 15q26.3, respectively) and schizophrenia, no significant associations have been found (Gunnell *et al.* 2007; Bonvicini *et al.* 2010). Regarding the insulin receptor substrates (IRSs) 1–4, linking both the IR and IGF-1R with intracellular pathways (Choi & Sung 2000; Lavan *et al.* 1997; White 1998; Xu *et al.* 1999), it is the *IRS-1* and *IRS-4* genes (located on chromosomes 2q36 and Xq22.3, respectively) that earlier have been investigated in relation to schizophrenia (Gunnell *et al.* 2007; Melkersson & Persson 2011; Melkersson *et al.* 2011). Associations between *IRS-4* SNPs and body mass index (BMI) of schizophrenia patients have been reported as well as one case of a patient with schizophrenia and a mutation in the *IRS-4* gene (Melkersson & Persson 2011; Melkersson *et al.* 2011), but between an *IRS-1* SNP and schizophrenia no association has been found (Gunnell *et al.* 2007). On the other hand, the *IRS-2* and *IRS-3* genes have – as yet – not been studied in schizophrenia. This would be worthwhile doing, especially to study the *IRS-3* gene.

The human *IRS*-3 gene is predicted by genome sequence analysis to be located on chromosome 7q22.1 (Glöckner *et al.* 1998; http://www.ensembl.org [release 48, December 2007]; http://www.ncbi.nlm.nih.gov [version 13.10.2011, build 37.3]). In earlier release of the database, it was indicated as a protein-coding gene consisting of five exons and four introns (http://www.ensembl.org [release 48, December 2007]), but since 2009 it is annotated as pseudogene (*IRS-3P*) (http://www.ncbi.nlm.nih.gov [version 13.10.2011, build 37.3]). Nevertheless, although pseudogenes have lost their ability to produce a functional protein, many of them are transcriptionally active and code RNAs that play critical roles in gene regulation (Pink *et al.* 2011). The IRS-3 protein has so far not been detected in humans, but in mouse and rat, where it in contrast to IRS-1 and IRS-2, exhibits more limited tissue expression at relatively lower levels (Björnholm *et al.* 2002; Giovannone *et al.* 2000; Kokk *et al.* 2005; Lavan *et al.* 1997; Lavan & Lienhard 1993; Milarski *et al.* 1995; Sciacchitano & Taylor 1997; Yen *et al.* 2004). Studies in mice have also shown that IRS-3 is expressed at the highest level early in embryonic development, although it persists at lower levels in postnatal life, suggesting that it may play an important role in mediating the actions of growth factors, in addition to its putative role in mediating the metabolic actions of insulin (Sciacchitano & Taylor 1997). Moreover, in contrast to IRS-1, IRS-2 and IRS-4, which predominantly are localized in the cytosol of the cell near to the plasma membrane, IRS-3 is localized also in the cell nucleus (Anai *et al.* 1998; Kabuta *et al.* 2002). Regarding potential functions of the IRS-3 protein, data obtained with embryonic fibroblast cell lines prepared from mice suggest that IRS-3 may act as a negative regulator of IGF-1 induced intracellular signalling by suppressing the function of IRS-1 and IRS-2 proteins at several steps (Tsuruzoe *et al.* 2001). Likewise, IRS-3 has been demonstrated to inhibit glucose/ IGF-1 induced IRS-2-mediated signalling in pancreatic beta cells, especially in the relatively dedifferentiated INS-1 cells that have tumorigenic (i.e. rat insulinoma) origin and show increased expression of the IRS-3 protein (Lingohr *et al.* 2003). Furthermore, results obtained with Chinese hamster ovary cells, overexpressing human IR and rat IRS-3, indicate that IRS-3 inhibits insulin-stimulated cell cycle progression (Kaburagi *et al.* 2004). On the other hand, data from studies on mouse and rat adipocytes suggest that both IRS-1, IRS-2 and IRS-3 play important roles in insulin-induced glucose transport in brown and white adipose tissues, but that IRS-3 predominantly is involved in regulating this process in the absence of IRS-1 or IRS-2 (Escribano *et al.* 2007;

Kaburagi *et al.* 1997; Zhou *et al.* 1999). Loss of IRS-1 in combination of IRS-3 interferes with formation of white adipose tissue, and the *Irs1*$^{-/-}$/ *Irs3*$^{-/-}$ double knock-out mice has severe lipoathrophy, diabetes and increased prenatal and early postnatal lethality (Laustsen *et al.* 2002). Contrary, a study in *IRS-3* null mice has shown that lack of IRS-3 alone does not effect normal growth, glucose homeostasis or fertility (Kahn & Saltiel 2005; Liu *et al.* 1999). Moreover, it is only IRS-3 of the four IRSs that has been found to work as a transcriptional regulator in the cell nucleus (Kabuta *et al.* 2002; 2010).

Assuming that these functions mediated by *IRS-3* in rodents resemble those mediated by *IRS-3* or *IRS-3P* in humans, this gene may play a role in the pathogenesis of brain and body abnormalities in schizophrenia, specially of those developmentally-related (Melkersson & Persson 2011; Melkersson *et al.* 2011; Meltzer 1976; Nilsson *et al.* 2006; Perrin *et al.* 2007; Ryan *et al.* 2003; Vita *et al.* 2000; Wozniak *et al.* 1993). Furthermore, a Finnish genome-wide scan provides evidence for linkage of schizophrenia to the chromosome 7q.22 region that is predicted to include the human *IRS-3* gene, and in a recent cytogenetic analysis, also chromosomal abnormalities associated with schizophrenia were identified within this 7q.22 region (Burada 2009; Ekelund *et al.* 2000). In this study, we therefore chose to study the *IRS-3* gene as a candidate gene in schizophrenia. We first screened the *IRS-3* gene sequence of patients and control subjects for DNA sequence variations and then carried out a case-control study.

PATIENTS AND METHODS

Patients and control subjects

Consecutive out-patients from psychiatric polyclinics in the region of Stockholm, Sweden and with the diagnosis of schizophrenia according to DSM-IV criteria (American Psychiatric Association 1994) were asked to participate in this study. In total 93 patients, 46 males and 47 females, gave their written informed consent to participate. The patients were structurally interviewed about mental and physical health in themselves and their relatives, and the patient group is described elsewhere in detail (Melkersson 2009). In brief, all patients were unrelated Caucasian individuals. They were in full or partial remission regarding psychotic symptoms, and were all receiving long-term therapy with antipsychotics. Their mean (s.d.) age was 44 (9) years, and their duration of schizophrenia illness ranged from 0.5 to 42 years [mean (s.d.) = 18 (9) years, median = 17 years]. Control subjects were 57 unrelated Caucasian individuals (16 males and 41 females) who lived in the Stockholm County or in the nearby Uppsala County and gave written informed consent to participate in the study. The control subjects were also interviewed about their own mental and physical health and also about that of their relatives. They were all healthy individuals with no family history of psychotic disorder and diabetes mellitus (DM) (type 1, type 2 or other types). Their mean (s.d.) age was 45 (11) years. The study was approved by the Ethics Committee of Karolinska Institutet, Stockholm, Sweden.

Determination of height and BMI

Height and weight were measured, and BMI was calculated as weight divided by the square of height (kg/m^2) (Labhart 1986).

Collection and DNA preparation of blood samples

Venous blood was taken in EDTA-containing tubes from all patients and control subjects and stored at –20 °C until preparation of DNA. Genomic DNA was extracted from peripheral blood leukocytes by using a Genomic DNA Purification Kit (Gentra Systems Inc., Minneapolis, MN, USA). The extracted DNA was frozen at –20 °C until sequenced.

DNA sequencing

The *IRS-3* gene on chromosome 7q22.1 was at study start year 2007 predicted to be located from position 100 164 721 to 100 168 025 (21; 22), and in this study, this whole gene region was DNA sequenced in all patients and control subjects. Genomic DNA was amplified by polymerase chain reaction (PCR), carried out in a Gene Amp® PCR System 2700 (Applied Biosystems, Foster City, CA, USA), followed by cleaning of the PCR products with Shrimp Alkaline Phosphatase and Exonuclease I (Fermentas International Inc., Burlington, Canada). Thereafter, the PCR fragments were sequenced in both directions, using BigDye® Terminator v3.1. sequencing kit (Applied Biosystems, Foster City, CA, USA), and analyzed by means of capillary electrophoresis in an ABI Prism 3730 Sequencer (Applied Biosystems, Foster City, CA, USA). Post-sequencing editing and alignment of sequences were made with the program Sequencher ™4.5 (Gene Codes Corporation, Ann Arbor, MI, USA).

Fragment analysis

To determine whether patients and control subjects were homozygous or heterozygous for the 16 nucleotide deletion/ insertion polymorphism in the *IRS-3* gene with accession number rs57426638 (Table 1), fragment size analysis was performed on fluorescently labelled PCR products in addition to the DNA sequencing. PCR products were diluted 1:100 in water and 1.5 µL of diluted samples were mixed with 10 µL HiDi Formamide and 0.1 µL GeneScan ™500Rox Size Standard (Applied Biosystems, Foster City, CA, USA). Then, samples were injected into an ABI 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA), where the different-sized fragments were separated by means of capillary electrophoresis. Fragment scoring was performed using the GeneMapper ™v4 software (Applied Biosystems, Foster City, CA, USA).

Tab. 1. Data regarding all polymorphisms in the insulin receptor substrate-3 gene[a].

Polymorphism id[a,b,c]	Polymorphism position[a] (NCBI build 37.3)	Polymorphism[a]	Reference[a]	Predicted gene location[d]
1. rs189354593	100 164 726	C/T	C/C	Exon 1
2. rs146322108	100 164 891	A/G	G/G	Intron 1
3. rs181003855	100 164 907	A/G	A/A	Intron 1
4. rs186464480	100 164 953	A/G	A/A	Intron 1
5. rs189772934	100 164 995	A/G	G/G	Intron 1
6. rs139311095	100 165 035	A/C	C/C	Intron1
7. rs149970944	100 165 130	A/C	A/A	Exon 2
8. rs61733851	100 165 171	C/T	C/C	Exon 2
9. rs181372905	100 165 217	G/T	G/G	Intron 2
10. rs148726709	**100 165 249**	**C/T**	**C/C**	**Intron 2**
11. rs144906243	100 165 269	C/T	C/C	Intron 2
12. rs2686814	100 165 503	C/T	C/C	Intron 2
13. rs113992587	100 165 508	A/C	A/A	Intron 2
14. rs77833347	100 165 777	A/G	G/G	Exon 3
15. SNP1/ rs185703140	**100 165 881**	**C/T**	**C/C**	**Intron 3**
16. rs34227160	100 165 951/ 100 165 952	–/G	–/–	Intron 3
17. rs2686813	100 166 068	C/G	G/G	Intron 3
18. rs142209639	100 166 111 – 100 166 126	–/CCCCGCCCCACGGCCC	–/–	Intron 3
19. rs73711134	**100 166 121**	**C/T**	**C/C**	**Intron 3**
20. rs57426638	**100 166 122 – 100 166 137**	**–/GGCCCCCCCGCCCCAC**	**–/–**	**Intron 3**
21. rs151085424	100 166 124	C/G	C/C	Intron 3
22. rs138266928	100 166 125	A/C	C/C	Intron 3
23. rs148278179	100 166 126	C/G	C/C	Intron 3
24. rs79199968	100 166 135 – 100 166 152	–/CCACGGCCCCCCCGCCCC	–/–	Intron 3
25. rs73407326	100 166 221	A/C	C/C	Intron 3
26. rs190555052	100 166 288	A/G	G/G	Intron 3
27. rs34437892	100 166 424	A/C	C/C	Intron 3
28. rs6966514	100 166 585	C/G	G/G	Exon 4
29. rs117078492	**100 166 597**	**A/G**	**G/G**	**Exon 4**
30. rs147962921	100 166 711	A/G	A/A	Intron 4
31. rs141716072	100 166 780	C/G	G/G	Intron 4
32. rs73407330	100 166 789	C/T	C/C	Intron 4
33. rs67147615	**100 167 002**	**C/T**	**T/T**	**Intron 4**
34. rs183143470	100 167 073	C/T	T/T	Intron 4
35. rs73156206 (suspect)[e]	100 167 138	C/G	G/G	Intron 4
36. rs147786169	100 167 142	A/G	G/G	Intron 4
37. rs117722415	**100 167 156**	**A/G**	**G/G**	**Intron 4**
38. rs75521004	**100 167 263**	**C/G**	**C/C**	**Intron 4**
39. rs61744581	**100 167 810**	**A/G**	**G/G**	**Exon 5**
40. rs11978967	**100 167 880**	**A/C**	**C/C**	**Exon 5**

[a]From the NCBI database (http://www.ncbi.nlm.nih.gov [version 13.10.2011, build 37.3]); [b]The polymorphic-distributed polymorphisms in our study population are written in extra bold type; [c]SNP1 refers to a novel SNP not earlier described in the dbSNP; [d]According to the Ensembl database December 2007 (http://www.ensembl.org [release 48, December 2007]); [e]Variation suspected to be false positive due to artifacts of the presence of a paralogous sequence in the genome or evidence suggested sequencing error or computation artifacts

Data analyses

Pairwise linkage disequilibrium (LD) and haplotypes were calculated using the PHASE program version 2.1. (Stephens & Scheet 2005; Stephens *et al.* 2001). Data are presented as mean and standard deviation (s.d.). Statistical differences in genotype, allele and haplotype frequencies between patients and control subjects or between subgroups of patients were examined with cross tables and Fisher's exact test. In comparison between different groups of genotypes or haplotypes regarding height and BMI of patients and control subjects, one-way analysis of variance (ANOVA) was employed, and when controlling for gender, a two-way ANOVA was performed. The criterion for significance for all tests was set at ≤0.05. The statistical analyses were performed using the statistical programs SAS version 9.1. (SAS Institute Inc., Cary, NC, USA) and Statistica 9.0 (Statsoft Inc., Tulsa, OK, USA).

RESULTS

The DNA sequence of the *IRS-3* gene in patients (n=93) and control subjects (n=57) was compared to the reference sequence of the gene (http://www.ensembl.org [release 48, December 2007]). Besides 36 SNPs, two 16-nucleotide polymorphisms and one 18-nucleotide polymorphism already described in the Ensembl and National Centre for Biotechnology Information (NCBI) databases (http://www.ensembl.org [release 48, December 2007]; http://www.ncbi.nlm.nih.gov [version 13.10.2011, build 37.3]), one novel SNP was revealed and registered by us (Table 1; SNP1/ rs185703140). Of these total 40 polymorphisms, 10 were polymorphic-distributed and 30 monomorphic-distributed in our study population (Tables 1 and 2). Close LD was found between rs67147615 and rs75521004, rs67147615 and rs11978967, and rs75521004 and rs11978967 (Figure 1).

Tab. 2. Genotype and allele frequencies regarding the 10 polymorphic-distributed polymorphisms in the insulin receptor substrate-3 gene in 93 schizophrenia patients (P) compared with 57 control subjects (C).

Polymorphism id[a]	Polymorphism[a]	Genotype frequencies (%)							Allele frequencies (%)[b]		
		P			C						
		1-1	1-2	2-2	1-1	1-2	2-2	*p*-value	P	C	*p*-value
10. rs148726709	C/T	97.85	2.15	0.00	100.00	0.00	0.00	0.53	98.92	100.00	0.53
15. SNP1/ rs185703140	C/T	100.00	0.00	0.00	98.25	1.75	0.00	0.38	100.00	99.12	0.38
19. rs73711134	C/T	100.00	0.00	0.00	98.25	1.75	0.00	0.38	100.00	99.12	0.38
20. rs57426638	–/GGCCCCCCCGCCCCAC	2.15	33.33	64.52	3.51	33.33	63.16	0.90	81.18	79.82	0.77
29. rs117078492	A/G	0.00	0.00	100.00	0.00	5.26[c]	94.74	**0.05**	100.00	97.37	**0.05**
33. rs67147615	C/T	0.00	10.75	89.25	0.00	14.04	85.96	0.61	94.62	92.98	0.62
37. rs117722415	A/G	0.00	3.23	96.77	0.00	5.26	94.74	0.67	98.39	97.37	0.68
38. rs75521004	C/G	88.17	11.83	0.00	85.96	14.04	0.00	0.80	94.09	92.98	0.81
39. rs61744581	A/G	0.00	1.08	98.92	0.00	0.00	100.00	1.00	99.46	100.00	1.00
40. rs11978967	A/C	0.00	13.98	86.02	0.00	14.04	85.96	1.00	93.01	92.98	1.00

SNP = single nucleotide polymorphism; [a]Same as in Table 1; [b]Only highest allele frequency is shown; [c]1 man and 2 women had A/G.

IRS-3 polymorphism[a]										
rs148726709	rs148726709									
SNP1/ rs185703140	0.000	SNP1/ rs185703140								
rs73711134	0.000	0.000	rs73711134							
rs57426638	0.002	0.001	0.001	rs57426638						
rs117078492	0.000	0.000	0.000	0.002	rs117078492					
rs67147615	0.000	0.052	0.052	0.015	0.001	rs67147615				
rs117722415	0.000	0.000	0.000	0.085	0.000	0.001	rs117722415			
rs75521004	0.000	0.049	0.049	0.016	0.001	**0.944**	0.001	rs75521004		
rs61744581	0.000	0.000	0.000	0.014	0.000	0.000	0.000	0.000	rs61744581	
rs11978967	0.001	0.044	0.044	0.018	0.001	**0.848**	0.002	**0.898**	0.000	rs11978967

Fig. 1. Pairwise linkage disequilibrium data indicated as r^2 values for the 10 polymorphic-distributed polymorphisms in the insulin receptor substrate-3 (*IRS-3*) gene. [a]Same id as in Table 1.

Tab. 3A. Estimated haplotypes, based on the 10 polymorphic-distributed polymorphisms in the insulin receptor substrate-3 gene, and their frequencies in the overall study population[a].

Haplotypes[b]	Frequency (%)
H1. CCC[+][c]GTGCGC	72.00
H2. CCC[–][d]GTGCGC	16.33
H3. CCC[+][c]GCGGGA	6.00
H4. CCC[–][d]GTACGC	2.00
H5. CCC[+][c]ATGCGC	1.00
HX. All rare[e]	<1.00

[a]n=150; [b]Alleles are numbered 10, 15, 19–20, 29, 33, 37–40 as in Table 1; [c][+] = insertion of [GGCCCCCCCGCCCCAC]; [d][–] = deletion of [GGCCCCCCCGCCCCAC]; [e]All haplotypes with estimated frequencies <1% in patients and control subjects

Tab. 3B. Frequencies of estimated haplotypes, based on the 10 polymorphic-distributed polymorphisms in the insulin receptor substrate-3 gene, in 93 schizophrenia patients (P) compared with 57 control subjects (C).

Haplotypes[a]	P vs C						*p*-value
	– /–	–/H	H/H	–/–	–/H	H/H	
H1. CCC[+][b]GTGCGC	3.23	45.16	51.61	7.02	49.12	43.86	0.41
H2. CCC[–][c]GTGCGC	70.97	26.89	2.15	66.67	31.58	1.75	0.81
H3. CCC[+][b]GCGGGA	89.25	10.75	0.00	85.96	14.04	0.00	0.61
H4. CCC[–][c]GTACGC	96.77	3.23	0.00	94.74	5.26	0.00	0.67
H5. CCC[+][b]ATGCGC	100.00	0.00	0.00	94.74	5.26[e]	0.00	**0.05**
HX. All rare[d]	93.55	6.45	0.00	96.49	3.51	0.00	0.71

[a]Alleles are numbered 10, 15, 19–20, 29, 33, 37–40 as in Table 1; [b][+] = insertion of [GGCCCCCCCGCCCCAC]; [c][–] = deletion of [GGCCCCCCCGCCCCAC]; [d]All haplotypes with estimated frequencies <1% in patients and control subjects; [e]1 man and 2 women had –/H5

Tab. 4A. Genotype groups of polymorphism rs57426638 in the insulin receptor substrate-3 gene in relation to height of 92 schizophrenia patients[a] and 57 control subjects.

Polymorphism - rs57426638				*p*-value[b]
Genotype group	[+]/[+][c]	vs	[+]/[–][c,d]+ [–]/[–][d]	
Patients (n)	59		33	
Height (m) Mean (s.d.)	1.73 (0.10)		1.74(0.10)	0.68 (0.83)
Control subjects (n)	36		21	
Height (m) Mean (s.d.)	1.74 (0.10)		1.68 (0.08)	**0.03 (0.05)**

[a]Data was missing in one patient; [b]*p*-values are given not corrected as well as corrected for gender in brackets; [c][+] = insertion of [GGCCCCCCCGCCCCAC]; [d][–] = deletion of [GGCCCCCCCGCCCCAC]

Tab. 4B. Haplotype 2 of the insulin receptor substrate-3 gene in relation to height of 92 schizophrenia patients[a] and 57 control subjects.

Haplotype 2. CCC[–][b]GTGCGC				*p*-value[c]
Haplotype group	–/–	–/H2	H2/H2	
Patients (n)	65	25	2	
Height (m) Mean (s.d.)	1.73 (0.09)	1.74 (0.11)	1.75 (0.14)	0.90 (0.94)
Control subjects (n)	38	18	1	
Height (m) Mean (s.d.)	1.74 (0.10)	1.68 (0.09)	1.57 (0.00)	**0.04 (0.03)**

[a]Data was missing in one patient; [b][–] = deletion of [GGCCCCCCCGCCCCAC]; [c]*p*-values are given not corrected as well as corrected for gender in brackets

Genotype, allele and haplotype frequencies regarding the 10 polymorphic-distributed polymorphisms in patients and control subjects, together with results of association analyses, are presented in Tables 2, 3A and 3B. Significant differences in genotype and allele frequencies were found for rs117078492 in that the frequencies of the A/G genotype and A allele of this SNP were increased in the control subjects compared to those in the patients (Table 2; p=0.05). Similarly, a significant difference in frequency of the haplotype 5, constructed from the 10 polymorphic-distributed polymorphisms and including this A allele, was found between patients and control subjects (Table 3B; p=0.05).

Significant associations were also found between height of control subjects, but not that of patients, and the polymorphism rs57426638 or haplotype 2 in the *IRS-3* gene (Tables 4A and B), whereas between BMI and *IRS-3* genotypes or haplotypes, no associations were found either in patients or control subjects (data not shown). Moreover, the estimated haplotype 3X, based on the two polymorphisms rs57426638 and rs117078492 that associated with height or schizophrenia, differed between patients and control subjects in that this haplotype was found only in the control subjects (Tables 5A and B; p=0.05).

Subgroup analysis of the patients alone showed no significant associations between family history of schizophrenia (presence/absence), subtype of schizophrenia (paranoid/non-paranoid) or DM and/ or family history of DM (presence/ absence) and *IRS-3* genotypes or haplotypes (data not shown).

DISCUSSION

In this study, based on DNA sequencing of the *IRS-3* gene on chromosome 7q22.1 from position 100 164 721 to 100 168 025, followed by case-control analyses of total 10 detected polymorphisms, a negative association between the A/G SNP rs117078492 at position 100 166 597 and schizophrenia was found. In addition, negative

Tab. 5A. Estimated haplotypes, based on rs57426638 and rs117078492 in the insulin receptor substrate-3 gene, and their frequencies in the overall study population[a].

Haplotypes[b]	Frequency (%)
H1X. [+][c]G	79.67
H2X. [–][d]G	19.33
H3X. [+][c]A	1.00

[a]n=150; [b]Alleles are numbered 20, 29 as in Table 1;
[c][+] = insertion of [GGCCCCCCCGCCCCAC]; [d][–] = deletion of [GGCCCCCCCGCCCCAC]

Tab. 5B. Frequencies of estimated haplotypes, based on rs57426638 and rs117078492 in the insulin receptor substrate-3 gene, in 93 schizophrenia patients (P) compared with 57 control subjects (C).

Haplotypes[a]	P vs C						*p*-value
	–/–	–/H	H/H	–/–	–/H	H/H	
H1X. [+][b]G	2.15	33.33	64.52	3.51	38.60	57.89	0.60
H2X. [–][c]G	64.52	33.33	2.15	63.16	33.33	3.51	0.90
H3X. [+][b]A	100.00	0.00	0.00	94.74	5.26[d]	0.00	**0.05**

[a]Alleles are numbered 20, 29 as in Table 1;
[b][+] = insertion of [GGCCCCCCCGCCCCAC]; [c][–] = deletion of [GGCCCCCCCGCCCCAC]; [d]1 man and 2 women had –/H3X

associations were found between the estimated haplotypes 5 or 3X including the A allele of this A/G SNP and schizophrenia. Taken together, these results point to a protective role for the A allele of this SNP against schizophrenia development.

The A/G genotype of this by prediction exon-4-located SNP is expected to change the amino acid coding, resulting in an altered IRS-3 protein in individuals carrying the A/G instead of G/G genotype. Since *IRS-3* in humans may be a gene that is transcribed at low levels, and/ or is transcribed only, for example, in embryonic tissues at certain times and in tumour tissues, it may be limited represented in EST databases (Glöckner *et al.* 1998). However, some human ESTs, originating from for example 1) whole brain of a 73 day old female infant (BX105833.1), 2) sciatic nerve (BQ943086.1), 3) anaplastic brain oligodendroglioma with chromosomes 1p/ 19q loss (BG820682.1), 4) insulinoma (BM272818.1) and 5) lacrimal gland (CK429601.1), were identified in the NCBI EST database (http://blast.ncbi.nlm.nih.gov) and determined to be cDNA encoding fragments (533, 904, 732, 446 and 630 base pair long, respectively) of the human *IRS-3* gene, giving some support for the existence of a functional human *IRS-3* gene and the expression of the human IRS-3 protein, especially in dedifferentiated cells, though not in all cell types (Björnholm *et al.* 2002; Ozyildirim *et al.* 2005; http://blast.ncbi.nlm.nih.gov). Since 2009, the *IRS-3* gene is indicated as pseudogene in the database (http://www.ncbi.nlm.nih.gov [version 13.10.2011, build 37.3]). However, several pseudogenes are transcribed to RNA and can be involved in gene regulation with importance for example in brain development and function (Pink *et al.* 2011; Presutti *et al.* 2006). So, although the role of the human *IRS-3* gene still remains to be clarified (Anai *et al.* 1998; Kabuta *et al.* 2002; Sciacchitano & Taylor 1997), it might be possible that an altered IRS-3 protein or altered *IRS-3* RNA in the brain exerts a protection against schizophrenia development through mediating an impaired intracellular inhibition on the actions of insulin and growth factors, resulting in a strengthening of insulin and growth factor-induced intracellular signalling, specially during the embryonic and early postnatal periods.

Interestingly, the 16-nucleotide polymorphism rs57426638 or the haplotype 2 including this polymorphism associated with height of the control subjects in that they showed to be shorter if they did not carry the 16-nucleotide insertion of this polymorphism. In contrast, these associations were lacking in the schizophrenia patients, who had unchanged height independent of carrying the 16-nucleotide insertion or not. Together, these findings point to a role for the *IRS-3* gene in growth regulation and support our hypothesis that impaired insulin/ IGF signalling in cells may underlie known abnormalities in schizophrenia such as growth dysregulation.

Through fine mapping of the DNA sequence of intron 3 in the *IRS-3* gene in our 150 patients and control subjects, the 16-nucleotide insertion/ deletion polymorphism rs57426638 located from 100 166 122

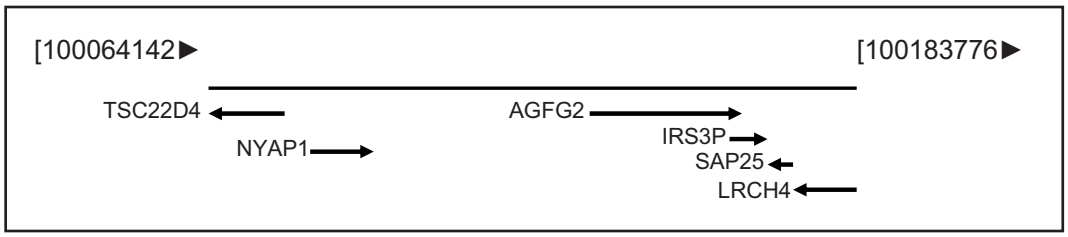

Fig. 2. Overlap between the 5′ upstream region of the insulin receptor substrate-3 (*IRS-3*) gene and the 3′ untranslated region of the Arf-GAP domain and FG repeats-containing protein 2 (*AGFG2*) gene, as reported in the most recent release of the database (http://www.ncbi.nlm.nih.gov [version 13.10.2011, build 37.3]).

to 100 166 137 was detected. However, the other 16-nucleotide polymorphism (rs142209639) and the 18-nucleotide polymorphism (rs79199968) in intron 3, which also are reported in the dbSNP, could not be detected. This difference between our results and the reports in the dbSNP may be explained by the fact that these two polymorphisms are rare and therefore not are present in our smaller groups of patients and control subjects or that the parts of intron 3 next to the 16-nucleotide polymorphism rs57426638, which include both rs142209639 and rs79199968, are difficult to DNA sequence and therefore wrongly reported into the dbSNP.

In the most recent release of the database (http://www.ncbi.nlm.nih.gov [version 13.10.2011, build 37.3]), the predicted location of the *IRS-3* gene is changed marginally from position 100 164 721 – 100 168 025 to position 100 162 778 – 100 167 851 according to the *IRS-3P* entry (NG_005538.4). At the same time, an unexpected overlap between the 5' upstream region of the *IRS-3* gene and the 3' untranslated region of the Arf-GAP domain and FG repeats-containing protein 2 (*AGFG2*) gene, coding the AGFG2 protein that is involved in maintenance and spread of immunodeficiency virus type 1 (HIV-1) infection, is reported in the database (Figure 2; Kahn *et al.* 2008; Panaro *et al.* 2011), pointing to that available information regarding the exact delimitation of the 5' end of the *IRS-3* gene is somewhat uncertain. The overlapping region ranges from position 100 162 778 to 100 165 842 and includes in all 3065 nucleotides (22). However, the region of the *AGFG2* gene that is reported to overlap with the *IRS-3* gene is a non-coding *AGFG2* region (http://www.ncbi.nlm.nih.gov [version 13.10.2011, build 37.3]). Neither did we find any polymorphisms in the DNA sequenced part of this overlapping region between *IRS-3* and *AGFG2* (i.e. position 100 164 721 – 100 165 842) differing in genotype and allele frequencies between our schizophrenia patients and control subjects. On the other hand, at the 3' end of the *IRS-3* gene, the nearest gene – the sin3A-associated protein 25kDa (*SAP25*) gene – is located about 2000 base pair downstream at position 100 169 855 – 100 171 270 (Figure 2). This gene codes the SAP25 protein which may play a regulatory role in repression of gene transcription (Shiio *et al.* 2006), and interestingly, this gene is located close enough to the *IRS-3* gene that polymorphisms in the two genes might be linked.

In the present study, the results with p-values ≤ 0.05 were considered statistically significant, although the results not would have obtained significance with correction for multiple testing, for example with Bonferroni correction. The main reasons for not using corrections for multiple testings in this study were the reasonably strong a priori hypothesis and the clearly exploratory nature of the study (Rothman 1990). The significant differences found in this study are also here reported for the first time, and therefore independent replication is the most proper way to test the robustness of our findings.

It might also be argued that our patient and control subject groups differed as regards occurrence of DM and a family history of DM, and that therefore our results might have been confounded. However, this can be ruled out since no differences were found between patients with or without DM and/ or family history of DM regarding their *IRS-3* genotypes or haplotypes.

Our findings of negative associations between schizophrenia and the SNP rs117078492 or the haplotypes 5 or 3X including this SNP in the *IRS-3* gene on chromosome 7q22.1 are in addition supported by earlier studies, providing evidence both for linkage of schizophrenia to the chromosome 7q22 region and for chromosomal abnormalities associated with schizophrenia at chromosome 7q21–7q22 (Burada 2009; Ekelund *et al.* 2000; Gordon *et al.* 1994; Idol *et al.* 2007; Knight *et al.* 2009; Yan *et al.* 2000).

Other researchers have also reported on possible susceptibility genes that function protectively in schizophrenia, such as those encoding the angiotensin-converting enzyme, the DISC1 interacting 14-3-3epsilon protein and the phosphodiesterase 4B enzyme (Crescenti *et al.* 2009; Ikeda *et al.* 2008; Pickard *et al.* 2007). However, in contrast to the protection by those genes that followed a genotype risk model in which the risk for schizophrenia decreased with the number of protective alleles involved (Crescenti *et al.* 2009; Ikeda *et al.* 2008; Pickard *et al.* 2007), our study showed 0% of the expectedly protective A allele of the SNP rs117078492 in the *IRS-3* gene in patients with schizophrenia, suggesting that the A allele of this SNP may confer a full protection against schizophrenia development.

In conclusion, our findings suggest that individuals carrying the A allele of the *IRS-3* SNP rs117078492 and the *IRS-3* haplotypes 5 and 3X including this A allele, have a protection against schizophrenia development.

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