Association Studies of HSPA1A and HSPA1L Gene Polymorphisms With Schizophrenia in the Polish Population

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Background. Schizophrenia is a severe psychiatric disorder with a strong genetic component. The HSP70 chaperones are particularly interesting in terms of schizophrenia, especially with regard to neurodevelopmental hypothesis, because they are critical regulators in normal neural physiological function as well as in cell stress responses.

Aim of the study. The present study aimed to determine whether genetic variants in the HSPA1A (rs1008438, rs562047) and HSPA1L (rs2075800) genes are associated with the risk of paranoid schizophrenia and the clinical presentation of the disease.

Methods. A total of 1080 unrelated Polish subjects of Caucasian origin (401 schizophrenia cases and 679 healthy controls) were recruited. Three single nucleotide polymorphisms (SNP) were genotyped using PCR-RFLP (rs562047) or TaqMan (rs1008438, rs2075800) assays. All analyses were conducted for the full sample and within subgroups stratified by gender.

Results. There were no statistically significant differences in genotype or allele distributions of all polymorphisms tested between the schizophrenia and control groups. We also failed to find any schizophrenia predisposing haplotype in the whole group. A sex-stratified analysis revealed haplotypic association with paranoid schizophrenia in men, albeit the risk effect was contributed only by a rare haplotypes. More importantly, rs562047 variant was significantly associated with PANSS total and PANSS negative scores in schizophrenia.

Conclusions. Our results support previously reported associations between HSPA1A and HSPA1B SNPs and schizophrenia symptomatology. Further population-based prospective studies with larger sample sizes from different ethnic groups should be performed to clarify the role of different HSP70 genes in the pathogenesis of schizophrenia. © 2018 IMSS. Published by Elsevier Inc.

Key Words: Association study, Paranoid schizophrenia, HSPA1A, HSPA1L, SNP, Haplotype.

Introduction

Schizophrenia is a common and severe psychiatric disorder caused by the interaction between genetic and environmental risk factors. Schizophrenia is both highly heritable and polygenic, with risk variants range in population frequencies from common to extremely rare (1–3). The largest published schizophrenia large-scale genome-wide association studies (GWAS), that from Schizophrenia Working Group of the Psychiatric Genomics Consortium (2), have identified 108 independent significant risk loci. More recently, in a meta-analysis combining a British study population with previous published 2014 GWAS, a 50 novel associated loci have been found (3). Notable associations, that support established hypotheses about the etiopathogenesis of schizophrenia, are enriched among genes involved in brain development and neuronal differentiation.
synaptic plasticity, dopaminergic, serotonergic, GABA-ergic, and glutamatergic neurotransmission, calcium channel function, and immunity (2–6).

Heat shock proteins (HSPs) are a family of highly conserved proteins classified according to their molecular weight into several classes: HSP100, HSP90, HSP70, HSP60, HSP40, and small HSPs (7). HSPs are expressed both constitutively and under stressful conditions such as hyperthermia, oxidative stress, or chemical stressors. They function as molecular chaperones necessary for the proper folding of polypeptides, refolding of misfolded proteins, translocation of proteins across membranes (cognate proteins), and in preventing protein denaturation and improper polypeptide aggregation during exposure to stress (inducible forms) (7).

The HSP70 chaperones are particularly interesting in terms of schizophrenia, especially with regard to neurodevelopmental hypothesis, because they are critical regulators in normal neural physiological function as well as in cell stress responses. The CNS critical developmental processes (i.e. rapid cell proliferation, migration and differentiation) are vulnerable to disruption following thermal or chemical stress. Therefore, it is hypothesized that defective production or function of HSP70 in response to embryonic insults may lead to the congenital brain insults found in schizophrenia (8). Evidence for the induction of HSP70 in the Central Nervous System (CNS) following different stressors and their neuroprotective properties against CNS damage have been extensively described (9,10). The over-expression of HSP70 have been proven to enhance protection of neurons against ischemic damage (11), and also against kainic acid- and glutamate- induced excitotoxicity (12). HSP70 are also understood to be involved in synaptic protection following exposure to stress facilitating normal neurotransmission (13). Moreover HSP70 are among the most potent suppressors of neurodegeneration in animal models. The relationships between HSP70 and neurodegenerative disorders have been extensively reviewed (7,9). A positive findings in association studies of HSPA1A and HSPA1B polymorphisms in neurodegenerative diseases include Alzheimer’s and Parkinson’s have also been published (14,15).

It is important to note that multiple associated risk variants emerging from GWAS of schizophrenia are within the major histocompatibility complex (MHC) region on chromosome 6p (2,16,17). The MHC region is very gene-dense, containing many genes with roles in immunity (18). Three genes of HSP70 family (HSPA1A, HSPA1B and HSPA1L) are mapped to the (MHC) class III region on chromosome 6p21.3. Several authors identified the antibodies against HSP70 in schizophrenic patients (19,20), which supports the immune mechanism of schizophrenia. In fact, schizophrenia and autoimmune disorders have several features in common like exacerbation of symptoms when exposed to stress. It is possible that neurodevelopmental aberrations of schizophrenia could be linked with immunoreactivity to HSP70 reducing their neuroprotective functions (21).

Studies that reported associations between HSP70 gene polymorphisms and schizophrenia are strongly limited. The first positive findings were obtained in a Korean population (21–23). More recently, we have demonstrated that HSP1A rs1043618 (+190G/C) and HSP1B rs1061581 (+1267A/G) polymorphisms may potentially contribute to susceptibility to paranoid schizophrenia and clinical presentation of the disease in Caucasian population (24). We have also found an association between HSPA1B gene polymorphisms and paranoid schizophrenia in the study involving four variants of the HSPA1B (rs2763979, rs6457452, rs539689, and rs9281590) (unpublished results).

In this study, we analyzed the three variants of the HSPA1A (rs562047, rs1008438) and HSPA1L (rs2075800) genes to extend and confirm previous findings of association between HSP70 gene polymorphisms and paranoid schizophrenia in larger samples.

Materials and Methods

Study Participants

The study population was consisted of 401 unrelated patients with a paranoid schizophrenia diagnosis (162 [40%] females and 239 [60%] males; mean age ± SD 41.3 ± 12.4, range 18–73) and 679 unrelated, ethnically matched controls (320 [47%] females and 359 [53%] males; mean age ± SD 40.4 ± 8.7, range 20–68). All enrolled participants were Caucasians of Polish origin living in Silesia region. The patients were recruited in the two centers: The Department and Clinic of Psychiatry, Medical University of Silesia in Katowice and the Neuropsychiatric Hospital in Lubliniec. The consensus diagnoses were made by two experienced psychiatrists according to DSM-IV-TR (Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision) criteria for schizophrenia. Inclusion was restricted to patients with paranoid schizophrenia. Exclusion criteria for patients involved any other major psychiatric disorders, neurological illness, endocrine disorders and autoimmune diseases. All patients were recruited during hospitalization for an acute or chronic schizophrenia. The mean duration of schizophrenia was 15.7 years (SD = 10.2, range 1–47). Symptom severity was assessed using the Positive and Negative Syndrome Scale (PANSS) (25), measured by a trained psychiatrists at the time of admittance. Factors were calculated using classic three-factor model of PANSS composed of 30 items divided into three subscales: positive (items P1–P7), negative (items N1–N7), and general psychopathology (items G1–G16), as well as using the five factor model of PANSS proposed by
van der Gaag et al. (26) comprises a positive factor (P1+P3+G9+P6+P5+G1+G12+G16-N5), a negative factor (N6+N1+N2+N4+G7+N3+G16+G8+G13-P2), a disorganised factor (N7+G11+G10+P2+N5+G5+G12+G13+G15+G9), an excitement factor (G14+P4+P7+G8+P5+N3+G4+G16), and an emotional factor (G2+G6+G3+G4+P6+G1+G15+G16), including all 30 PANSS items. The age of schizophrenia onset was the age at first manifestation of positive symptoms. The mean age of onset was 25.6 years (SD = 6.8, range 13–54). Data on the age of onset, family mental health history, and suicidal behaviour were collected from medical records and through personal interviews with patients. The healthy controls were selected from volunteer blood donors of the Regional Centre of Blood Donation and Treatment in Katowice. Exclusion criteria for controls were: any neurological disorders, chronic and acute physical illness such as infections, autoimmune or allergic diseases, current psychiatric problems, family history of mental disorders, history of psychiatric medication, psychiatric hospitalization, suicide attempts, and history of substance abuse or dependency (data obtained through questionnaire and interview). All the subjects have provided written consent after receiving a complete explanation of the project. The Bioethics Committee of Medical University of Silesia approved the study (No. KNW/0022/KBi/38/I/12).

**SNP Selection and Genotyping**

A total of three SNPs (rs562047 G/C, rs1008438 A/C [HSPA1A], and rs2075800 C/T [HSPA1L]) were selected to be examined according to the following criteria: a) the minor allele frequency ≥10% in the European population (SNP information was retrieved from the National Center for Biotechnology Information, dbSNP, [http://www.ncbi.nlm.nih.gov/SNP/](http://www.ncbi.nlm.nih.gov/SNP/)). SNPs with a low MAF are rare (the minor allele frequency was reported to have associations with other related disorders (15,27), and d) the SNPs (rs1008438) were reported to have associations with other related disorders in previous studies (15).

The venous blood of patients and controls was collected in 5 mL tubes, and the DNA was extracted from peripheral leukocytes using a QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA). Quality of DNA extracts was checked electrophoretically using a 1.5% agarose gel stained with ethidium bromide. The concentration of DNA was estimated by spectrophotometric measurement using a BioPhotometer plus (Eppendorf AG, Hamburg). Sample of low DNA quality or concentration has been removed from the genotyping.

Genotypes of rs562047 polymorphism were determined by a PCR-RFLP method. The primer sequences were as follows: Forward, 5'-GCCGACAGAGAGCCGGAA-3' and Reverse, 5'-CCTCGGATCTTTCTAC-3'. The PCR cycling conditions were as follows: initial denaturation at 94°C for 5 min, subsequent denaturation at 94°C for 30 s, annealing for 1 min at 57°C and extension for 1 min at 72°C, for 35 cycles, with a final elongation at 72°C for 10 min. Amplification was performed using G-Storm GS1 thermal cycler (Gene Technologies LTD, Essex, UK) with the Taq polymerase (Epiconcet, Biotechnologies) according to the manufacturer’s protocol, in a reaction mixture total volume of 25 μl. The PCR amplified products were digested with Eco31I endonuclease (Thermo Fisher Scientific) and distinctive restriction digest fragments (421 bp for C allele, 346 bp and 75 bp for G allele) were detected by electrophoresis in 3% agarose gels.

Genotyping of rs1008438 (HSPA1A) and rs2075800 (HSPA1L) SNPs was performed using an allele-specific TaqMan assay on a CFX96 real-time PCR detection system (Bio-Rad), in a 96-well format. Each reaction contained 10 ng DNA, 12.5 μl TaqMan Universal PCR Master Mix, 1.25 μl Assay Mix (Life Technologies), and nuclease free water to bring the final reaction volume of 25 μl. The PCR thermal cycling was as follows: initial denaturing at 95°C for 10 min; 40 cycles of denaturation at 95°C for 15 sec and annealing/extension at 60°C for 1 min. Each 96 well plate contained 91 samples of an unknown genotype, two blank controls (no-template control), and three positive control samples for each genotype. For rs1008438 polymorphism, the TaqMan primers were: 5'-GCCGTGTGGTCAGGAA-3' and 5'-GCTTGCCGGAAATAT-3', while probes were FAM-AGGCAGAAAACCCCTTG-MGB for C and VIC-AGGCAGAAAACCCCTTG--MGB for A allele (28). The catalog number for rs2075800 C/T polymorphism is C_3052613_1.

For quality control, approximately 5% of the samples were randomly selected and re-tested to evaluate the genotype accuracy by both genotyping methods. The concordance among duplicated samples was 100%. Samples with missing genotypes have been removed from the analysis.

**Statistical Analyses**

Statistical analyses were performed using STATISTICA 10.0 PL (StatSoft, Cracow, Poland), StataSE 13.0 (StataCorp LP, TX, U.S.) and R software. Statistical significance was set at a *p* value below 0.05. All tests were two-tailed. Imputations were not done for missing data. Nominal and ordinal data were expressed as percentages, whilst interval data were expressed as mean value ± standard deviation. Differences in the allele, genotype and haplotype frequencies between schizophrenia...
Table 1. Genotype and allele frequencies of the HSPA1A and HSPA1L polymorphisms in patients (n = 401) and controls (n = 679)

<table>
<thead>
<tr>
<th>SNP</th>
<th>Total</th>
<th>Schizophrenia</th>
<th>Controls</th>
<th>χ²</th>
<th>p</th>
<th>Schizophrenia</th>
<th>Controls</th>
<th>χ²</th>
<th>p</th>
<th>Schizophrenia</th>
<th>Controls</th>
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<tr>
<td>C/C</td>
<td>144 (36)</td>
<td>257 (38)</td>
<td>1.88</td>
<td>0.391</td>
<td></td>
<td>61 (38)</td>
<td>129 (40)</td>
<td>0.61</td>
<td>0.735</td>
<td>83 (35)</td>
<td>128 (36)</td>
<td>1.42</td>
<td>0.493</td>
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<tr>
<td>C/T</td>
<td>200 (50)</td>
<td>311 (46)</td>
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<td></td>
<td></td>
<td>79 (49)</td>
<td>144 (45)</td>
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<td></td>
<td>121 (51)</td>
<td>167 (47)</td>
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<tr>
<td>T/T</td>
<td>57 (14)</td>
<td>111 (16)</td>
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<td></td>
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<td>22 (14)</td>
<td>47 (15)</td>
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<td></td>
<td>35 (15)</td>
<td>64 (18)</td>
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<tr>
<td>A/A</td>
<td>163 (41)</td>
<td>285 (42)</td>
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<td>66 (41)</td>
<td>133 (42)</td>
<td>1.37</td>
<td>0.504</td>
<td>97 (41)</td>
<td>152 (42)</td>
<td>0.55</td>
<td>0.758</td>
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<td>146 (44)</td>
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<td>111 (46)</td>
<td>156 (43)</td>
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<tr>
<td>C/C</td>
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<td>92 (14)</td>
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<td>27 (17)</td>
<td>41 (13)</td>
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<td>37 (13)</td>
<td>51 (14)</td>
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<tr>
<td>G/G</td>
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<td>510 (75)</td>
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<td>0.759</td>
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<td>121 (75)</td>
<td>234 (73)</td>
<td>3.63</td>
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<td>184 (77)</td>
<td>276 (77)</td>
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<tr>
<td>C/G</td>
<td>87 (22)</td>
<td>157 (23)</td>
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<td>36 (22)</td>
<td>83 (26)</td>
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<td>51 (21)</td>
<td>74 (21)</td>
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<tr>
<td>C/C</td>
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<td>5 (3)</td>
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<tr>
<td>C</td>
<td>488 (61)</td>
<td>825 (61)</td>
<td>0.01</td>
<td>0.876</td>
<td></td>
<td>201 (62)</td>
<td>402 (63)</td>
<td>0.06</td>
<td>0.814</td>
<td>287 (60)</td>
<td>423 (59)</td>
<td>0.15</td>
<td>0.697</td>
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<td>T</td>
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<td>486 (36)</td>
<td></td>
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<td></td>
<td>123 (38)</td>
<td>228 (36)</td>
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<td></td>
<td>191 (40)</td>
<td>295 (41)</td>
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<tr>
<td>A</td>
<td>506 (63)</td>
<td>872 (64)</td>
<td>0.27</td>
<td>0.601</td>
<td></td>
<td>201 (62)</td>
<td>412 (64)</td>
<td>0.51</td>
<td>0.476</td>
<td>305 (64)</td>
<td>460 (64)</td>
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<tr>
<td>C</td>
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<td>486 (36)</td>
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<td>123 (38)</td>
<td>228 (36)</td>
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<td>173 (36)</td>
<td>258 (36)</td>
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<td>rs562047</td>
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<tr>
<td>G</td>
<td>697 (87)</td>
<td>1117 (87)</td>
<td>0.02</td>
<td>0.876</td>
<td></td>
<td>278 (86)</td>
<td>551 (86)</td>
<td>0.02</td>
<td>0.902</td>
<td>419 (88)</td>
<td>626 (87)</td>
<td>2.11</td>
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</tr>
<tr>
<td>C</td>
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<td>181 (13)</td>
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<td>59 (12)</td>
<td>92 (13)</td>
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</tbody>
</table>

N (%)

Patients and healthy controls were assessed using the χ² test, and the maximum likelihood χ² test. Distribution of variables was evaluated by the Shapiro-Wilk test and quantile-quantile (Q–Q) plot, and homogeneity of variables was assessed by the Levene test. The Hardy-Weinberg Equilibrium was assessed by the Fischer’s exact test. The extent of the linkage disequilibrium (LD) expressed in terms of the D’ and r² coefficients and haplotypes were estimated using the SNPStats. For each SNP, multiple inheritance models (co-dominant, dominant, recessive, over-dominant and log-additive) were tested to assess potential association with schizophrenia risk and the best fitting models were determined by using Akaike information criterion (AIC) and Bayesian information criterion (BIC). The differences between genotypes, sex and scores of PANSS subscales and age of onset were examined using two-way ANOVA with Tukey’s post-hoc test. Comparisons of PANSS factors between patients with and without suicide attempt were performed with t-Student test for independent samples.

Results

There was a difference in age between males and females (38.5 ± 11.9 vs. 45.5 ± 12.1, p < 0.001) in the study group. There was no difference in age between males and females among the controls (40.6 ± 9.2 vs. 40.3 ± 8.2, p = 0.65). There was a small difference in the percentage of females between study and control groups (40.4 vs. 47.1%; χ² = 4.62, p < 0.05).

The genotype distributions of 3 SNPs did not depart significantly from the Hardy-Weinberg Equilibrium (HWE) both in the patients [rs2075800 [p = 0.40], rs1008438 [p = 0.45], rs562047 [p = 0.38]], as well as in the controls [rs2075800 [p = 0.30], rs1008438 [p = 0.40], rs562047 [p = 0.99]].

Genotype and allele frequencies of the three studied SNPs are presented in Table 1. There were no statistically significant differences in genotype and allele distributions between schizophrenia patients and controls for all studied polymorphisms both in the entire groups and when the groups were subdivided according to gender. No

Table 2. The results of haplotype analysis with the three SNP markers (rs2075800 – rs1008438 – rs562047) in patients and controls

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Frequency</th>
<th>OR (95% CI)</th>
<th>p</th>
<th>Frequency</th>
<th>OR (95% CI)</th>
<th>p</th>
<th>Frequency</th>
<th>OR (95% CI)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>T—A—G</td>
<td>0.3853</td>
<td>1.00</td>
<td>-</td>
<td>0.3702</td>
<td>1.00</td>
<td>-</td>
<td>0.3974</td>
<td>1.00</td>
<td>-</td>
</tr>
<tr>
<td>C—A—G</td>
<td>0.2467</td>
<td>0.99 (0.79–1.24)</td>
<td>0.93</td>
<td>0.2589</td>
<td>0.87 (0.62–1.22)</td>
<td>0.42</td>
<td>0.2366</td>
<td>1.14 (0.84–1.55)</td>
<td>0.41</td>
</tr>
<tr>
<td>C—C—G</td>
<td>0.2338</td>
<td>1.10 (0.88–1.38)</td>
<td>0.40</td>
<td>0.2309</td>
<td>1.04 (0.73–1.47)</td>
<td>0.85</td>
<td>0.2365</td>
<td>1.16 (0.86–1.56)</td>
<td>0.34</td>
</tr>
<tr>
<td>C—C—C</td>
<td>0.1264</td>
<td>0.96 (0.72–1.28)</td>
<td>0.79</td>
<td>0.1332</td>
<td>1.02 (0.66–1.56)</td>
<td>0.94</td>
<td>0.1206</td>
<td>0.93 (0.64–1.37)</td>
<td>0.73</td>
</tr>
<tr>
<td>rare</td>
<td>0.0078</td>
<td>2.50 (0.76–8.20)</td>
<td>0.13</td>
<td>0.0068</td>
<td>0.45 (0.05–4.15)</td>
<td>0.48</td>
<td>0.0089</td>
<td>9.57 (1.16–78.72)</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

OR, odds Ratio; CI, confidence interval.
Significant p values <0.05 are in bold.
associations were also found when analysis was performed in the subgroups of patients with a positive and negative family history of schizophrenia.

Five genetic models of inheritance (co-dominant, dominant, recessive, over-dominant, log-additive) were also used to evaluate the potential association between individual polymorphisms and schizophrenia risk. Unfortunately, we did not find statistically significant difference for each polymorphic site in any genetic model tested. The results were also non-significant after the stratification according to gender (data not shown).

The linkage disequilibrium (LD) analysis showed a strong linkage for all pairs of markers: rs2075800 and rs1008438 (D’ = 0.9872, r² = 0.3568, p < 0.001), rs2075800 and rs562047 (D’ = 0.8940, r² = 0.0787, p < 0.001), rs1008438 and rs562047 (D’ = 0.9292, r² = 0.2322, p < 0.001).

As demonstrated in Table 2, the haplotype frequencies did not differ between schizophrenia and controls in the whole groups. After stratification according to gender, only the rare haplotypes were significantly associated with paranoid schizophrenia in men (OR = 9.57, p < 0.05).

To investigate the association between HSPA1A (rs562047, rs1008438) and HSPA1L (rs2075800) polymorphisms and age of onset and severity of symptoms measured by PANSS two-way ANOVA (sex × genotype) was performed. As shown in Table 3, there was a significant interaction between sex and rs562047 genotype for PANSS total score. A Tukey’s post-hoc test showed significant differences between men and women who carried the C/C genotype (p < 0.05). In the classic three-factor model of PANSS a significant association was found between sex and rs562047 genotype for PANSS negative score. Post-hoc Tukey’s correction revealed significant differences between men and women with the C/C genotype (p < 0.01), as well as between the C/G and C/C men carriers (p < 0.05), and G/G and C/C men carriers (p < 0.01). There was also a trend towards significance for an interaction between sex and rs1008438 genotype for PANSS negative score (p = 0.078). When taking into account the PANSS factor solutions of van der Gaag et al. (26), two SNPs exhibited a statistical trend in the PANSS emotional (rs1008438) and excitement factors (rs562047), respectively. We have observed that women carrying the rs1008438 C/C genotype had higher mean PANSS emotional scores than men with the same genotype (23.7 vs. 20.3). Similar differences were also detected across rs562047 genotypes (C/C women 24.0 vs. C/C men 17.5), but the differences failed to reach statistical significance.

We further investigate the association between HSPA1A and HSPA1L polymorphisms and suicidal behavior in the entire sample and after stratification according to gender. None of the three SNPs tested were found to be associated with suicidal risk. However we found that disorganized and emotional PANSS factor scores, and age of onset were significantly associated with suicidal risk. Patients who attempted suicide had lower mean PANSS disorganized scores (30.2 vs. 32.1, p < 0.05), higher mean PANSS emotional scores (23.8 vs. 21.7, p < 0.01), and had the first episode of schizophrenia at a younger age (23.9 vs. 26.0, p < 0.05) in comparison with non-suicidal attempters.

Discussion

This study focuses on the analysis of the influence of three SNPs of HSPA1A and HSPA1L genes on susceptibility to paranoid schizophrenia and clinical presentation of the disease in the Polish population. Our study is among the few published studies that evaluated the associations between SNPs of HSPA1A and HSPA1L and risk of schizophrenia development. In our first study (24), we examined the association between HSPA1A rs1043618 (+190G/C), HSPA1B rs1061581 (+1267A/G) and HSPA1L rs2227956 (+2437T/C) SNPs and paranoid schizophrenia. The results we obtained have revealed the first evidence that the HSPA1A variants may increase the risk for developing paranoid schizophrenia in Caucasian Polish residents in a sex-dependent manner. We also demonstrated the effects of HSPA1A genotypes on psychopathology and age of onset.

In the current experiment, the sample size was larger than those of previous study, and included the samples of previous study. However three other SNPs were tested in this study (rs1008438 and rs562047 in HSPA1A, and rs2075800 in HSPA1L) compared to previous report. It should be emphasized that rs1008438 and rs2075800 SNPs were examined for their association with schizophrenia for the first time. The functional significance of rs1008438 (−110A/C) SNP has been previously analyzed. Wu YR, et al. (15) have found the influence of rs1008438 SNP on transcriptional activity of HSPA1A (higher activity by the presence of −110A allele). We would like to speculate that potentially functional rs1008438 polymorphism, similarly to rs1043618 polymorphism, may increase the risk of schizophrenia affects the synthesis level of the HSP70-1a protein and in consequence impairs the cellular response to stress. However our larger case-control analysis showed no association in genotype/allele frequency of rs1008438 and rs562047 polymorphisms in HSPA1A with schizophrenia, which is inconsistent with the result of our previous study showing significant association in allele and genotype at HSPA1A rs1043618 SNP between paranoid schizophrenia and controls. The most likely explanation for these disparate findings is the difference in sample size between these two studies. The rs562047 SNP was also analyzed in schizophrenia on a Korean population (21,23). Although Kim et al. (21) showed no impact of rs562047 on the susceptibility to schizophrenia, they found haplotype based association with A-C-C-G haplotype being more represented among patients. We failed to find any
Table 3. Results from the two-way ANOVA (sex, genotype) on Positive and Negative Syndrome Scale (PANSS) and age of onset in patients with schizophrenia

<table>
<thead>
<tr>
<th>Variable</th>
<th>Sex</th>
<th>Genotype</th>
<th>Sex × genotype</th>
<th>Sex</th>
<th>Genotype</th>
<th>Sex × genotype</th>
<th>Sex</th>
<th>Genotype</th>
<th>Sex × genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age of onset</td>
<td>0.102</td>
<td>0.680</td>
<td>0.054</td>
<td>0.712</td>
<td>0.529</td>
<td>0.084</td>
<td>0.670</td>
<td>0.506</td>
<td>0.246</td>
</tr>
<tr>
<td>Total PANSS</td>
<td>&lt; 0.05</td>
<td>0.233</td>
<td>0.390</td>
<td>&lt; 0.01</td>
<td>0.264</td>
<td>0.130</td>
<td>&lt; 0.01</td>
<td>0.997</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Positive scale</td>
<td>&lt; 0.05</td>
<td>0.398</td>
<td>0.153</td>
<td>0.146</td>
<td>0.078</td>
<td>0.527</td>
<td>0.116</td>
<td>0.803</td>
<td>0.568</td>
</tr>
<tr>
<td>Negative scale</td>
<td>0.303</td>
<td>0.521</td>
<td>0.131</td>
<td>&lt; 0.05</td>
<td>0.880</td>
<td>0.078</td>
<td>&lt; 0.001</td>
<td>0.694</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>General psychopathology scale</td>
<td>&lt; 0.05</td>
<td>0.257</td>
<td>0.575</td>
<td>&lt; 0.01</td>
<td>0.318</td>
<td>0.144</td>
<td>&lt; 0.05</td>
<td>0.851</td>
<td>0.152</td>
</tr>
<tr>
<td>Positive factor</td>
<td>0.926</td>
<td>0.874</td>
<td>0.340</td>
<td>0.949</td>
<td>0.648</td>
<td>0.877</td>
<td>0.303</td>
<td>0.109</td>
<td>0.187</td>
</tr>
<tr>
<td>Negative factor</td>
<td>0.762</td>
<td>0.960</td>
<td>0.233</td>
<td>0.112</td>
<td>0.567</td>
<td>0.067</td>
<td>0.328</td>
<td>0.819</td>
<td>0.695</td>
</tr>
<tr>
<td>Disorganized factor</td>
<td>0.907</td>
<td>0.557</td>
<td>0.256</td>
<td>0.838</td>
<td>0.939</td>
<td>0.810</td>
<td>0.594</td>
<td>0.193</td>
<td>0.332</td>
</tr>
<tr>
<td>Excitement factor</td>
<td>0.453</td>
<td>0.449</td>
<td>0.152</td>
<td>0.961</td>
<td>0.356</td>
<td>0.407</td>
<td>0.238</td>
<td>0.060</td>
<td>0.145</td>
</tr>
<tr>
<td>Emotional factor</td>
<td>0.345</td>
<td>0.626</td>
<td>&lt; 0.05</td>
<td>&lt; 0.05</td>
<td>0.891</td>
<td>0.094</td>
<td>&lt; 0.05</td>
<td>0.654</td>
<td>0.212</td>
</tr>
</tbody>
</table>

Nominally significant p values are bold.

Van der Gaag’s model.

Mean total PANSS scores were: women-G/G 88.8 ± 17.8, C/G 85.6 ± 14.8, C/C 75.2 ± 11.3; men-G/G 90.7 ± 17.4, C/G 93.7 ± 16.1, C/C 103.5 ± 28.1.

Mean negative PANSS scores were: women-A/A 24.4 ± 6.5, A/C 25.2 ± 6.0, A/A 23.0 ± 5.7; men-A/A 25.3 ± 6.2, A/C 25.1 ± 6.3, A/A 27.2 ± 5.4.

Mean positive PANSS scores were: women-G/G 24.9 ± 6.2, C/G 23.8 ± 6.1, C/C 20.0 ± 4.5; men-G/G 25.0 ± 6.0, C/G 26.6 ± 6.1, C/C 33.3 ± 5.9.

Mean emotional PANSS factor scores were: women-A/A 22.1 ± 4.8, A/C 22.7 ± 4.7, A/A 23.7 ± 5.6; men-A/A 22.1 ± 5.0, A/C 21.8 ± 5.0, A/A 20.3 ± 3.7.

Mean emotional PANSS factor scores were: women-G/G 22.4 ± 4.9, C/G 23.1 ± 5.1, C/C 24.0 ± 2.6; men-G/G 21.8 ± 4.9, C/G 21.7 ± 4.7, C/C 17.5 ± 4.2.

schizophrenia predisposing haplotype in the whole groups, and after stratification according to gender only the rare haplotypes were significantly associated with paranoid schizophrenia in men, which limits the current finding in the direction of a possible false-positive finding. These results should also be interpreted with caution because of the small sample size. Interestingly, we previously found an impact of gender on the risk of schizophrenia for HSPA1A rs1043618 polymorphism (nearly 2 fold higher risk for women with the rs1043618 C/C genotype than for men with the rs1043618 C/C genotype) (24). Moreover our latest research focuses on HSPA1B polymorphisms revealed that C allele of rs539689 polymorphism contributes to paranoid schizophrenia only in males (unpublished results). A number of gender-specific genetic associations with schizophrenia risk have been also reported for other genes such as ZNF804A (29), the myelin transcription factor 1 like (MYT1L) (30), and interferon γ (IFN-γ) (31).

Unlike HSPA1A, HSPA1B exhibit constitutive expression, but exact function and transcriptional regulation of this gene are currently unknown (21). The HSPA1B rs2075800 SNP leads to a novel amino acid at residue 602 of HSP70-1t protein within the domain that modulates substrate binding and interaction with co-chaperones. It is conceivable that this non-synonymous SNP may affect the expression and functional efficiency of HSP-70-1t (27).

There were no significant differences in the allelic or genotype frequencies of the HSPA1L rs2075800 polymorphism between the schizophrenia patients and the controls in this study. These results confirmed previous findings of our group that HSP70 gene polymorphisms do not contribute to schizophrenia development in the Polish population (24). On the contrary rs2075799 in HSPA1L showed strong association and a power of distribution to schizophrenia in a Koran population, and T→A haplotype composed of the two HSPA1L SNPs (rs2227956 and rs2075799, respectively) was also significantly associated with schizophrenia (21). The discrepancies between results from different studies may reflect differences across populations in the contribution of HSP70 gene polymorphisms to schizophrenia.

Our study revealed that genetic variations of HSPA1B influence the severity of symptoms in paranoid schizophrenia. We found rs562047 significantly associated with PANSS total score, and PANSS negative score, and women carrying the rs562047 C/C genotype had significantly lower mean PANSS total (75.2 vs. 110.2) and negative scores (20.0 vs. 33.3), respectively, than men with the same genotype. We also observed a trend towards significant associations between rs1008438 and PANSS subscores. Interestingly, we had previously found an association between HSPA1A rs1043618 and HSPA1B rs1061581 and PANSS positive scores. Heterozygous genotypes were associated with higher scores in both sexes in comparison with homozygous genotypes for the minor allele (rs1043618 C/C and rs1061581 G/G) (24). We also found the influence of HSPA1B rs539689 and rs9281590 genotypes on psychopathology of schizophrenia. Carriers of rs539689 G/G genotype had significantly higher mean scores of positive, general, and total PANSS than heterozygous and homozygous C/C patients. For rs9281590, men who carried the homogenous genotype for the minor allele had higher mean...
scores of general PANSS than women with the same genotype (unpublished results). Pae et al. (23) have studied the impact of the rs562047 and 4 other SNPs of HSP70 genes on clinical presentation of the disease and drug response in schizophrenic patients. They demonstrated two haplotypes (A-C-G-G, and G-C-G-G) significantly associated with the difference in PANSS total and negative subscales from baseline to discharge, and speculated that HSP gene mutations may differentially contribute to negative and positive symptoms in accordance with the molecular functions of these proteins.

The HSPA1A and HSPA1L are mapped to the MHC region on the short arm of chromosome 6 (6p21.3−22.1), which is one of the most polymorphic and gene dense region in the human genome containing many genes with putative immune functions. The current understanding suggests that many of the genes within the MHC region not only play significant roles in immunity, but also in non-immune processes related to development, regulation etc. Multiple MHC association studies have shown association with schizophrenia several genes representing classical HLA (HLA-A, -B, -C, -DRB, -DQA, -DQB), as well as other genes like TNF-α or NOTCH4. The association of some alleles such as HLA-A9, HLA-A10, HLA-DRB1, and HLA-DQB1 were successfully replicated in different ethnic populations [reviewed by Debnath et al. (32)]. Because of the strong and variable LD existing across the MHC region, we cannot exclude that the associations found between HSP70 gene polymorphisms and schizophrenia are secondary to closely linked variations. In fact, Schroeder et al. (33) noted the LD between HSPA1B and TNF-α. The strong association between HLA-DR3 and polymorphisms in the regulatory region of the HSPA1A was also reported (34).

HSPs have distinct but overlapping functions, and much evidence indicates that their neuroprotective actions in the CNS may interfere. The overexpression of HSP27, similarly to HSP70, was found to protect neurons against ischemic damage (35) and excitotoxicity mediated by kainic acid (36). The presence of HSP27 and HSC70 (apart from HSP70) at the synapse following hyperthermia supports the hypothesis of their role in synaptic protective mechanisms during time of stress (13,37). Ishima T, et al. (38) have found significantly increased levels of HSP90α in cultured cells after treatment with aripiprazole (atypical antipsychotic drug, approved for the treatment of schizophrenia) and speculated that this protein may promote NGF-induced neurite outgrowth. Much of the research to date has focused on individual genes, whereas searching for associations between various susceptibility genes of HSP and schizophrenia may contribute to better understanding of the role of heat shock proteins in the pathogenesis of schizophrenia.

The present study has several limitations. (I) Our sample size seems to be modest compared to other studies in this field, and replication using larger sample sizes is necessary. A larger sample size would likely increase the statistical power of our results, and would allow us to evaluate the joint effects in stratified analysis. (II) We focused on only three SNPs of the HSP70 genes, thereby providing incomplete coverage of those genes, and we cannot exclude the possibility that other polymorphisms in the candidate genes may account for the association with schizophrenia through linkage disequilibrium. (III) Our haplotype analysis revealed only a rare haplotypes associated with schizophrenia in men, which limits the current finding in the direction of a possible false-positive finding. (IV) We did not investigate the effect of SNPs on gene function, so we were unable to assess the mechanism via which rs562047 C/C genotype is associated with the psychopathology of schizophrenia measured with PANSS. However, in contrast to many other association studies, our population was highly homogenous with respect to ethnicity (Caucasians of Polish origin), geographic regions, and schizophrenia subtype which minimizes the possible biases related to population stratiﬁcation. Nonetheless, none of the patients in this study were newly diagnosed (mean duration of schizophrenia was 15.7 years). Their inpatient status minimizes discrepancy in diagnosis and clinical measures. Thus, we have a modest but clear and well selected sample, which might be preferable compared to ethnically mixed but larger samples. Moreover, all analyses, we performed, were stratified according to gender. Gender differences in schizophrenia with respect to age of onset, clinical presentation, and course of illness are well-described in the research literature (39).

In conclusion, we have analyzed HSPA1A (rs562047, rs1008438) and HSPA1L (rs2075800) polymorphisms in paranoid schizophrenia patients of Polish Caucasian origin. Contrary to our previous studies, we found that no single SNP was significantly associated with schizophrenia risk. We also failed to find any schizophrenia predisposing haplotype in the whole group. Significant findings from sex-stratified haplotype analysis are limited by rare haplo- typic association with paranoid schizophrenia. However, one SNP, rs562047, was significantly associated with PANSS total and PANSS negative scores. Further additional population-based prospective studies with larger sample size from different ethnic groups should be performed to clarify the role of different HSP70 genes in the pathogenesis of schizophrenia.

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Conflict of Interest
We have no conflict of interest to declare.


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