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Title: Association of candidate Single Nucleotide Polymorphisms Related to Candidate Genes in an Iranian sample of Patients with Schizophrenia

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Abstract:

Background: Schizophrenia is a chronic heterogenic mental disorder. It is also a polygenic disorder by various molecular pathways. Many genes interfere in development of SCZ. All four genes, NrCAM, PRODH, ANK3, and ANKK1 were previously reported to be in association with Schizophrenia. The NrCAM contributes to creating cognitive deficiencies through the CAM’s signaling pathway (Zhang, Yu et al. 2015). PRODH plays important role in creating SCZ negative symptoms through the signaling pathway of glutamatergic and through the NMDA receptor. ANK3 affects ion channel and molecular adhesion in ranvier and initial segments of axons, leading to mental retardation, sleep disorder and SCZ. ANKK1 encodes a protein kinase and was reported to be associated with alcohol addiction, Attention Deficit Hyperactivity Disorder (ADHD) and SCZ.

Objective: This study aimed to investigate the association between candidate SNPs and variants of four genes, NrCAM, PRODH, ANK3, and ANKK1 and SCZ in an Iranian sample of patients with schizophrenia.

Method: The subjects were selected from Schizophrenic patients referring to Psychiatric Ward of Imam-Hussein Hospital and Schizophrenic Patients Support Institution (AHEBBA). 95 (30 Schizo Affective patients, 57 Paranoid patients and 8 disorganized) patients were recruited as the subjects in present case-control association study. 120 healthy subjects were recruited from the staff of the Tehran Medical Genetics Laboratory and a group of students from Islamic Azad University of Science and Research in Tehran. The genotypes were determined with molecular genotyping techniques of PCR-RFLP, ARMS-PCR and Cycle sequencing. Results were analyzed by Chi-Square test to investigate significant differences between cases and controls.

Results: The incidence of schizophrenia was 68% and 32% among men and women, respectively. The evaluation of allelic association between schizophrenia and all candidate SNPs, showed a significant association between, NrCAM's SNP rs10235968 and SCZ (P=0.001). Incidence of the allele-C among schizophrenic patients and healthy group was 66.3% and 50%, respectively, while the incidence of allel-T among the patients and healthy groups was 33.7% and 50%, respectively. No association was found between other candidate SNPs and SCZ among the subjects.

Keywords: SNP, NrCAM, PRODH, ANKK1, ANK3, Schizophrenia.
1. Introduction

Schizophrenia (SCZ) is a chronic mental disorder. Many genes and environmental factors are involved in SCZ development. NrCAM is a candidate gene for schizophrenia and has been reported in many studies with conflicting results (Ayalew, Le-Niculescu et al. 2012). NrCAM encodes neuron cell’s adhesion protein molecule and is located on 7q31.1. The Northern Blotting technique showed a 2kb transcript of NrCAM in all brain tissues. The 7.0 kb transcript of NrCAM is highly expressed in the brain’s Medula, Adrenal and Adrenal cortex (Lane, Chen et al. 1996, Wang, Williams et al. 1998). NrCAM owns 36 exons and is involved in WNT and CAM's signaling pathways and L1CAM interactions (Chen and Zhou 2010, Zhang, Yu et al. 2015). NrCAM is one of the twelve genes that are active in CAM's pathway and have significant relationship with schizophrenia. NrCAM acts as a neuron-neuron connection and a signal transmission in CAM's pathway (Zhang, Yu et al. 2015). The CAM's signaling pathway is a candidate pathway involved in SCZ, which has been repeatedly reported to be linked with both psychosis and neurocognitive dysfunctions (Zhang, Yu et al. 2015). CAM's pathway plays an important role in the cognitive functions of the brain, including formation of memory, attention, learning, reasoning, and thinking, which is disrupted in SCZ (Hargreaves, Anney et al. 2014). The gene NrCAM is active in neuron-neuron, neuron-glial adhesion and growth cone motility (Kamiguchi and Lemmon 1997). NrCAM has also been demonstrated to take part in many cellular processes of central and peripheral nervous systems, inclusive neurite growth, exon routing, myelination and cellular migration (Kamiguchi and Lemmon 1997). Using GWAS and signaling pathways data, researchers reported several signaling pathways contributing in the development of SCZ, including ionic channel pathway (Askland, Read et al. 2012), myelination pathway (Yu, Bi et al. 2014), apoptosis factor pathway, adhesion molecule pathway, growth factor signaling pathway and glutamate metabolism pathway (Jia, Wang et al. 2010). Cell adhesion molecules are glycoproteins expressed on cell surface and significantly contribute in biological processes including immune responses, inflammation responses, embryonic development (Elangbam, Qualls Jr et al. 1997). NrCAM is mostly reported with regard to autism (Sakurai, Ramoz et al. 2006). NrCAM's SNPs rs3763463, rs10235968, rs6967368 are located in the promoter area, and rs1269634 is located in intron area. They were evaluated among an Iranian sample of patients with schizophrenia. An association study on NrCAM's SNPs and SCZ was conducted in Korean population and no association was found between NrCAM's SNPs and SCZ (Kim, Kim et al. 2009). In a study conducted at the University of California, NrCAM SNP rs646558, showed association with SCZ (Atz, Rollins et al. 2007). association between NrCAM SNPs and personality disorder, addiction to amphetamines was evaluated by Yoo et al. Their results showed significant association between NrCAM SNP rs129634 and symptomatic amphetamine addiction (Yoo, Shim et al. 2012).

One of the most powerful risk factors for SCZ is 22q11 deletion syndrome (Levinson, Duan et al. 2011). Of all three patients with 22q11 deletion syndrome (which is also called DiGeorge or Velocardiofacial syndrome) one person is suffering from Schizophrenia/Schizoaffective (Pulver, Nestadt et al. 1994), while...
prevalence ratio of 22q11 deletion syndrome among the populations is 1/4000, this rate is about 1% among schizophrenic cases (Christofolini, Bellucco et al. 2011). 22q11 deletion syndrome was proposed as genetic kind of SCZ; therefore, all the sequences located in the 22q11 deletion area are addressed to be linked with schizophrenia. PRODH is one of the genes located on the chromosome 22q11.2 and includes 15 exons. PRODH encodes Proline Dehydrogenase enzyme. Catalyzing conversion of Proline into Glutamate. Hyperprolinemia is regarded to be involved in development of SCZ (Jacquet, Raux et al. 2002, Kempf, Nicodemus et al. 2008). On one hand, Proline is a modulator of glutamine neurotransmitter (Liu, Heath et al. 2002). Glutamatergic deficiency, in particular hypofunction of NMDA receptor, is also involved in development of SCZ (Galderisi, Merlotti et al. 2015). The administration of NMDA antagonist results in cognitive deficiency and SCZ syndromes among healthy people, leading to changes in dopaminergic neurons transmission (Galderisi, Merlotti et al. 2015). The association of PRODH SNP rs238731 located in the exon 12, and SCZ is supported in numerous studies (Shashi, Berry et al. 2009, Ota, Bellucco et al. 2014).

ANKK1 encodes a serine threonine kinase protein and belongs to a family of receptor-interacting serine/threonine-protein kinases which contribute in cell division and cell differentiation (Hamosh, Scott et al. 2002)(Jasiewicz, Samochowiec et al. 2014). ANKK1 is located on the chromosome 11q32.2 and includes 10 exons. ANKK1 was reported to be associated with alcohol addiction, eating disorder, SCZ and Attention Deficit Hyperactivity Disorder (ADHD) (Jasiewicz, Samochowiec et al. 2014) (Arab and Elhawary 2015). ANKK1 c.562C>T located in exon 3, was argued to have significant association with susceptibility to SCZ (Shirzad, Beiraghi et al. 2017). ANK3 is located on the chromosome 10q21.2, includes 52 exons and was reported to be associated with mental retardation and SCZ (Iqbal, Vandeweyer et al. 2013). The protein encoded by ANK3 is assumed to be connected to integral proteins and is mostly located in Ranvier node and axons. (Iqbal, Vandeweyer et al. 2013). ANK3 c.7649 G>T located in the exon 13 was shown to influence SCZ susceptibility (Shirzad, Beiraghi et al. 2017).

Materials and Methods

2.1. Patients and controls: This study was a case-control association study where none of the patients were relatives. A total of 95 patients (men=65, women=30) with average age 32 ±12.18 were recruited from Psychiatric ward of Imam Hussein Hospital and Schizophrenic Patient Support Institution (AHEBBA) in Tehran from August 2015 to May 2016. (30 Schizo Affective patients, 57 Paranoid patients and 8 disorganized patients). The patients’ demographic specifications including gender, age, place of birth and age at onset were recorded. Patients were identified by drawing pedigree and interview to recognize any physical illness or a family history of psychiatric disorders. Patients were clinically diagnosed with SCZ by an expert psychiatrist, according to the approach of Diagnostic and Statistical Manual of Mental Disorders (DSM–5) symbolism. Positive and Negative Syndrome Scale (PANSS), was used to recognize severity of Positive and Negative Syndromes among schizophrenic cases. A total of 120 healthy subjects (men=78, women=42) with an average age 35 ±14.14 were recruited from the staff of Tehran Medical Genetics
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Laboratory and a group of students from Islamic Azad University of Science and Research in Tehran. These subjects were demographically matched by age and gender. They were identified by drawing pedigree and interview to recognize any previous psychological or a family history of psychiatric disorders and physical diseases. Case and control groups were matched in terms of age and gender. None of the participants had a certain physical illness. The specifications of case and control subjects are introduced in Table 1.

Table 1: Demographics and characteristics of cases and controls

<table>
<thead>
<tr>
<th>Variable</th>
<th>SCZ patients</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>Frequency (%)</td>
<td>N</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td>95</td>
</tr>
<tr>
<td>Female</td>
<td>30</td>
<td>31.4 %</td>
</tr>
<tr>
<td>Male</td>
<td>65</td>
<td>68.6 %</td>
</tr>
<tr>
<td>Age</td>
<td>95</td>
<td>32 ±12.1</td>
</tr>
<tr>
<td>Age at onset</td>
<td>80</td>
<td>21.43 ±7.32</td>
</tr>
<tr>
<td>Family History (%)</td>
<td>80</td>
<td>80 ±10.0039</td>
</tr>
</tbody>
</table>

SCZ: schizophrenia, N: sample size, PANSS: positive and negative syndrome scale. As it can be seen, 65 males and 30 females were participated in the case group with average age 32 ± 12.18, PANSS score with average score 80 ±10.0039, as well as age at onset with average age 21.43 ±7.32 And 31.1% of patients had Family History of schizophrenia disorder. Also, among the controls group, 78 males and 42 females were participated with average age (35 ±14.14).

2.2. Ethical considerations: consent Letter was taken from all patients or their caregivers. This research was conducted in agreement with the Declaration of Helsinki. This investigation was authorized by the medical ethics committee of Science and Research Branch of Islamic Azad University, Tehran, Iran.

2.3. DNA extraction: The blood samples (5 ml) were obtained from all cases and controls in EDTA tubes and were prepared for DNA extraction. In this study, MagCore HF16 Automatic Nucleic Acid Extractor system (RBC Bioscience Corp, Taiwan) with MagCore blood Genomic DNA Extraction Kit (RBC Bioscience Corp, Taiwan) was used to extracting of Genomic DNA samples and then they were stocked in a temperature of -20°C. Nanodrop and 1% agarose gel electrophoresis were used to specified quality and quantity of the purified DNA.

2.4. Primer design: The software Gene Runner, (www.SNPs.com) and primer-blast-NCBI-NIH databases were used to designing primers for PCR-RFLP, ARMS-PCR and Cycle-Sequencing methods.

SNPs rs3763463, rs2238731 and c.562 C>T, were genotyped through PCR-RFLP method (Table 2). PCR-RFLP primers were designed according to the recognition site and the fragments length after enzymatic digestion. Remaining SNPs rs1269634, rs6967368, rs10235968, and c.7649 G>T were genotyped through ARMS-PCR method (Table3). Two forward primers and one reverse common primer were designed for any polymorphisms which were examined by ARMS-PCR (i.e. wild type [Wt] and mutant [mut]). A mismatch
would be made in the 3′ terminal of mut primer. However, more changes inside the last five nucleotides at the 3′ terminal of mut primer will lead to enhance the specificity (Little 1995)(Table3). A section of β-globin gene was chosen as internal control. Necessary primers for Cycle sequencing were designed for all polymorphisms and were listed in Table 4.

**Table 2: list of primers used for PCR-RFLP method.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP</th>
<th>Primer sequences (5′-3′)</th>
<th>Product length (bp)</th>
<th>Restriction Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRCAM</td>
<td>rs3763463 (C/G)</td>
<td>Forward: 5’...GCAGCAAGCAGTGTGTTTACTC...3’</td>
<td>320 bp</td>
<td>sstII</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5’...CTTCGAAATTCATCAGTTGGG......3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRODH</td>
<td>rs2238731 G&gt;T</td>
<td>Forward: 5’...GGACAGAGGTGGAGGCCC......3’</td>
<td>315 bp</td>
<td>Hin1II (NlalII)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5’...GTGATGGGGTCCTCATAGCC....3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANKK1</td>
<td>c.562 C&gt;T</td>
<td>Forward: 5’...ACCCCTGAAACAGGCAGATGC...3’</td>
<td>444 bp</td>
<td>Dde I</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5’...GTTCACACAGTCGCCAGGAAG...3’</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 3: list of primers used for ARMS-PCR method**

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP</th>
<th>Primer sequences (5′-3′)</th>
<th>Product length (bp)</th>
<th>Tm °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRCAM</td>
<td>rs1269634 (A/G)</td>
<td>Forward (Wt): 5’...GTCTAGTAATTTTCATGCGG...3’</td>
<td>260 bp</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Forward (Mut): 5’...GTCTAGTAATTTTCATGCGG...3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse(C): 5’...TCATAAGGATGGTAGACAGATTTG...3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NRCAM</td>
<td>rs6967368 (A/T)</td>
<td>Forward(Wi): 5’...CTCTTTTCATTGGGAAACCCT...3’</td>
<td>331 bp</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Forward Mut: 5’...CTCTTTTCATTGGGAAACCCT...3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse(C): 5’...CATGAGGAAGGAGAGCTACAG...3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NRCAM</td>
<td>rs10235968 (C/T)</td>
<td>Forward(Wi): 5’...CTCTCTTCGTGAGCCTAC...3’</td>
<td>126 bp</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Forward (Mut): 5’...CTCTCTTCGTGAGCCTAC...3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse(C): 5’...AAGGCTCCCGTGAGCCTAC...3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANK3</td>
<td>c.7649 G&gt;T</td>
<td>Forward(Wi): 5’...CATCCACATGGCATTTTAGAC...3’</td>
<td>471 bp</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Forward (Mut): 5’...CATCCACATGGCATTTTAGAC...3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse(C): 5’...GAGTCATTCCTCTTTTAC...3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β- globin gene (Internal control)</td>
<td>Forward Control primer: 5’...CAATGTACATGCCTTCCTGCAG...3’</td>
<td>800 bp</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse Control primer: 5’...GAGTCAGGGCTGAGAGATCACGGA...3’</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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Table 4: list of primers used for Cycle Sequencing method.

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNPs</th>
<th>Primer sequences (5’-3’)</th>
<th>Product length (bp)</th>
<th>Tm</th>
</tr>
</thead>
</table>
| NrCAM     | rs1269634 (A/G) | Forward: 5’... GGATGGTAGACAGATTATGCTCA... 3’  
Reverse: 5’... GCAGTTCAAGTGATGATAATGC... 3’ | 515 bp             | 58 |
|           | rs6967368 (A/T) | Forward: 5’... AATCTGCTCCTAACTTATCTCCATT... 3’  
Reverse: 5’... AAATGGTCCTCAGAAGTGAATTT... 3’ | 236 bp             | 59 |
|           | rs3763463 (C/G) | Forward: 5’... GCAGCAACAGTGAGTTATC... 3’  
Reverse: 5’... CTTCGAAATTCATCAGTTG... 3’ | 320 bp             | 60 |
|           | rs10235968 (C/T) | Forward: 5’... TGCTGGAGCTGACAAATGTT... 3’  
Reverse: 5’... ATTTCGTTTTACAAATAGGGGAAGTA... 3’ | 353 bp             | 60 |
| PRODH     | rs2238731 G/T | Forward: 5’... GGACAGAGTTGGAGGC... 3’  
Reverse: 5’... GTTGATGGGGTCCTCATAGCC... 3’ | 315 bp             | 61 |
| ANKK1     | c.562 C>T | Forward: 5’... ACCCTGGAACAGGC... 3’  
Reverse: 5’... GTTCACACAGTCCCAGGCAAG... 3’ | 444 bp             | 61 |
| ANK3      | c.7649 G>T | Forward: 5’... GGGTCTGATAAGCGG... 3’  
Reverse: 5’... ACCATTTTTAGGGCGTGCC... 3’ | 366 bp             | 59 |

2.5. PCR-AFLP: while Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) was used for genotyping target SNPs, needs to be digested using certain restriction enzymes. their Recognition site and the length of fragments obtained from the enzymatic digestion are described in Table 5.

Table 5: Restriction Enzymes and their Recognition site

<table>
<thead>
<tr>
<th>SNP</th>
<th>Restriction Enzyme</th>
<th>recognition site</th>
<th>the length of the fragments obtained from the enzymatic digestion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Wild homozygote</td>
</tr>
<tr>
<td>rs 2238731</td>
<td>Hin1 II (Nia III)</td>
<td>5’... C A T G ▼ 3’</td>
<td>317nt.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3’... ▼ G T A C ▲</td>
<td>5’</td>
</tr>
<tr>
<td>rs 3763463</td>
<td>SacII</td>
<td>5’... CC GC↓G</td>
<td>168nt, 152nt</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3’... GG↑CG CC... 5’</td>
<td></td>
</tr>
<tr>
<td>rs 897218854</td>
<td>Ddel</td>
<td>5’...C↓TNAG......3</td>
<td>182nt, 16nt, 246nt</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3’...GANT↑C.......5’</td>
<td></td>
</tr>
</tbody>
</table>
SNP: single nucleotide polymorphism.

Samples were evaluated for wild and mutant alleles in each PCR run. Two tubes containing positive and negative control were replicated along with other tubes. The appropriate restriction enzymes in agreement with manufacturer’s instructions were used to digesting the PCR products. 12% polyacrylamide gel electrophoresis and silver staining protocols were used to visualizing digested products.

**PCR conditions for PCR-AFLP:**
- Reaction volume: 20μl
- PCR mix: 19.05μl
- Primer concentration: 0.15μl (each forward and reverse)
- Taq polymerase: 0.15 μl
- Template DNA: 0.5μl (~16.5ng)

**Thermal cycling:**
- Primary denaturation: 5 minute at 95 °C
- No. of cycles: 30
- Denaturation: 30 seconds at 94°C
- Annealing: 30 seconds at 61°C
- Extension: 30 seconds at 72°C
- Terminal extension: 10 minute at 72°C

**Enzymatic digestion Conditions for digestion of PCR products directly after amplification:**
- Add:
  - PCR reaction mix: 5 μl
  - Nuclease-free water: 4.7 μl
  - 10x Buffer G: 1 μl
  - Restriction enzyme: 0.3μl
- Mix softly and spin down for a few second
- Incubate at 37 °C for over night
- Thermal inactivation: Restriction enzyme is inactivated by incubation at 65°C for 20 min.

**Electrophoresis:** 10 X 10 cm 12% polyacrylamide, 1 hour at 150 volts.

**Staining:** 0.1% silver nitrate.

2.6. **ARMS-PCR:** ARMS-PCR was performed in triplicates for all specimens. The results showed Wt allele or Mut allele depending on the amplicon bands detected on polyacrylamide gel. Samples carrying the Wt/Wt alleles or Mut/ Mut alleles showed a single band on polyacrylamide gel while two bands with same molecular size were expected for samples carrying both Mut and Wt alleles. A true negative is suggested by applying for replicating the internal control region and lack of amplification through the ARMS primers. 12% polyacrylamide gel electrophoresis and silver staining protocols were used to visualizing Amplified products.

**PCR conditions for ARMS-PCR:**

---

6 Wild type allele
7 Mutant type allele
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- Reaction volume: 20μl
- PCR mix: 18.21μl
- Primer concentration: 0.25μl (each forward (Mut/Wt) and reverse), 0.07 μl (each forward and reverse for beta globine)
- Taq polymerase: 0.15 μl
- Template DNA: 1μl (~13ng)

Thermal cycling:
- Primary denaturation: 5 minute at 95 °C
- No. of cycles: 30
- Denaturation: 30 seconds at 94°C
- Annealing: 30 seconds at 65°C
- Extension: 30 seconds at 72°C
- Terminal extension: 10 minute at 72°C

Electrophoresis: 10 X 10 cm 12% polyacrylamide, 1 hour at 150 volts.

Staining: 0.1% silver nitrate.

2.7. Sequencing: in order to confirm the results obtained by ARMS-PCR and PCR-AFLP, Applied Biosystem incorporation (ABI) and 3130 Genetic Analyzer was used for sequencing of one third of samples.

PCR conditions for Sequencing:
- Reaction volume: 20μl
- PCR mix: 19.05μl
- Primer concentration: 0.15μl (each forward and reverse),
- Taq polymerase: 0.15 μl
- Template DNA: 0.5μl (~20ng)

Thermal cycling:
- Primary denaturation: 5 minute at 95 °C
- No. of cycles: 30
- Denaturation: 30 seconds at 94°C
- Annealing: 30 seconds at 65°C
- Extension: 30 seconds at 72°C
- Terminal extension: 10 minute at 72°C

The PCR Products were transferred to advanced genomic department of Tehran Medical Genetic Laboratory for cycle sequencing. The cycle sequencing was implemented based on Applied Biosystem.

Applied Biosystem Protocol:
- 96°C for 1 min
- 25 cycles of:
  - 96 °C for 10 seconds.
  - 50 °C for 5 seconds.
  - 60 °C for 4 minute
  - 4 °C - hold

8 http://www.geneticlab.ir/
EDTA-Ethanol protocol was used for purifying products. After adding 12µl Formamid, purified products were denatured at 95°C for 5 minutes and then cool off to 4°C. Finally, products were run using 3130 Genetic Analyzer.

2.8. Statistical analysis:

The Chi-Square test was used to determine departure from Hardy–Weinberg Equilibrium (HWE) for all SNPs (Wittke-Thompson, Pluzhnikov et al. 2005, Rodriguez, Gaunt et al. 2009). To evaluating dates normality distributions, the Kolmogorov-Smirnov test was used. For analyzing the results, the Statistical Package for the Social Sciences version 24 (SPSS, Version 24; SPSS Inc., Chicago, IL) was used, to investigate the association of the candidate SNPs with SCZ. Non-parametric Pearson χ² test with a Bonferroni-corrected statistical significance level was used to evaluate allelic and genotypic frequencies of all candidate SNPs. Allele homozygosity and heterozygosity were specified OR (Odds Ratio) and 95% CI (Confidence Interval) were determined for all genotypes. Calculated Probability P-value ≤ 0.05 was regarded significant.

2. Results

Genotype and allele frequencies of all candidate SNPs: The genotype distribution for all candidate SNPs, among case and control did not deviate from those predicted by Hardy–Weinberg (p>0.05), except for SNP rs10235968 [controls: χ²=5.93, df =1, P=0.025]. The Chi-square for genotype distribution was calculated for all candidate SNPs, and listed in Tables 6. Incidence of allele-C and allele-T for NrCAM's SNP rs10235968 among patients was 66.3% and 33.7%, respectively, while the incidence of allele C and T was equal among healthy group. Genotyping frequency of two genotypes CC and TT for SNP rs10235968 among patients were 80.4% and 19.6% respectively while the frequencies for the two genotypes among healthy group were equal. The genotype and allele frequencies of all candidate SNPs were presented in Table 7.

Table 6: Genotype distribution for all candidate SNPs in the study

<table>
<thead>
<tr>
<th>gene</th>
<th>SNPs</th>
<th>χ² (P value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NrCAM</td>
<td>rs10235968</td>
<td>0.13(P&gt;0.05)</td>
</tr>
<tr>
<td></td>
<td>SCZ</td>
<td>5.93(P=0.025)</td>
</tr>
</tbody>
</table>
Association of SNPs with SZ

<table>
<thead>
<tr>
<th>Gene</th>
<th>Polymorphisms</th>
<th>N</th>
<th>Genotype frequency (%)</th>
<th>Allele frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Genotype frequency (%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CC</td>
<td>CT</td>
</tr>
<tr>
<td>NrCAM</td>
<td>rs10235968</td>
<td>95</td>
<td>41 (43.2)</td>
<td>44 (46)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>114</td>
<td>22 (18.5)</td>
<td>70 (59.7)</td>
</tr>
<tr>
<td>NrCAM</td>
<td>rs1269634</td>
<td>94</td>
<td>16 (16.8)</td>
<td>50 (52)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>118</td>
<td>16 (13.4)</td>
<td>56 (47.1)</td>
</tr>
<tr>
<td>NrCAM</td>
<td>rs6967368</td>
<td>95</td>
<td>71 (74.7)</td>
<td>20 (21.1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>116</td>
<td>91 (77.3)</td>
<td>21 (17.6)</td>
</tr>
<tr>
<td>NrCAM</td>
<td>rs3763463</td>
<td>95</td>
<td>76 (80)</td>
<td>18 (18.9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>117</td>
<td>93 (79)</td>
<td>24 (20.2)</td>
</tr>
<tr>
<td>PRODH</td>
<td>rs2238731</td>
<td>95</td>
<td>93 (97.9)</td>
<td>2 (2.1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>117</td>
<td>114 (97.5)</td>
<td>2 (1.7)</td>
</tr>
</tbody>
</table>

SNP: single nucleotide polymorphism. Chi-square’s exact P-value for genotype distribution of allele C for rs10235968 showed deviate from those predicted by the Hardy-Weinberg equilibrium in control group. The rest of candidate SNPs did not pervert from Hardy-Weinberg equilibrium in both patients and healthy subjects.

Table 7: Genotype and allele frequencies of all candidate SNPs/Mutations
3.2. Allelic Association: According to allelic comparisons, allele C for NrCAM SNP rs10235968 showed a significant association with SCZ [rs10235968 (C-allele): P =0.001, df =1, OR =1.969, 95% CI =1.323–2.929 after Bonferroni correction (adjusted p-value =0.001)]. The confidence interval (CI) for the odds ratio (OR) spanned above 1. The allelic frequency for NrCAM SNP rs10235968 (T/C) between non-schizophrenic samples was 0.5/0.5 respectively; whereas, between schizophrenic patients, was (0.33/0.66) respectively. The allelic frequency for the rest of candidate SNPs, displayed no significant difference between two patient and control groups.

[ NrCAM SNP rs126934: P =0.144, df =1, OR =1.407, 95% CI =0.88–2.22, rs6967368: P =0.593, df =1, OR =0.831, 95% CI =0.42–1.63, rs3763463 P =0.781, df =1, OR =0.817, 95% CI =0.05–13.28], [PRODH SNP rs2238731: P =0.881, df =1, OR =0.81, 95% CI =0.05–13.02], [ANKK1 SNP rs897218854: P =0.272, df =1, OR =0.989, 95% CI =0.96–1.01]. The brief results for association between candidate SNPs and SCZ are presented in Table 8.

Table 8: Association between candidate SNPs and schizophrenia

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chr</th>
<th>SNP/Mutation</th>
<th>A1/A2</th>
<th>Common allele</th>
<th>P Value</th>
<th>OR (95% CI)</th>
<th>Pearson χ²</th>
</tr>
</thead>
<tbody>
<tr>
<td>NrCAM</td>
<td>7</td>
<td>rs10235968</td>
<td>C/T</td>
<td>C</td>
<td>0.001</td>
<td>1.969 (1.323-2.929)</td>
<td>11.284</td>
</tr>
<tr>
<td>NrCAM</td>
<td></td>
<td>rs126934</td>
<td>A/G</td>
<td>A</td>
<td>0.144</td>
<td>1.407 (0.88-2.22)</td>
<td>2.134</td>
</tr>
<tr>
<td>NrCAM</td>
<td></td>
<td>rs6967368</td>
<td>T/A</td>
<td>T</td>
<td>0.593</td>
<td>0.831 (0.42-1.63)</td>
<td>0.285</td>
</tr>
<tr>
<td>NrCAM</td>
<td></td>
<td>rs3763463</td>
<td>G/C</td>
<td>C</td>
<td>0.781</td>
<td>0.817 (0.05-13.28)</td>
<td>0.078</td>
</tr>
<tr>
<td>PRODH</td>
<td>22</td>
<td>rs2238731</td>
<td>T/C</td>
<td>C</td>
<td>0.881</td>
<td>0.81 (0.05-13.02)</td>
<td>0.022</td>
</tr>
<tr>
<td>ANKK1</td>
<td>11</td>
<td>rs897218854</td>
<td>C/T</td>
<td>C</td>
<td>0.272</td>
<td>0.989 (0.96-1.01)</td>
<td>1.2</td>
</tr>
<tr>
<td>ANK3</td>
<td>10</td>
<td>c.7649G&gt;T</td>
<td>G/T</td>
<td>G</td>
<td>0.272</td>
<td>0.989 (0.96-1.01)</td>
<td>1.2</td>
</tr>
</tbody>
</table>

OR: odds ratio, CI: confidence interval, Chr: chromosome, SNP: single nucleotide polymorphism, A1/A2: allele1/allele2, Allele C for SNP rs10235968 suggests significant association with SCZ according to Pearson χ² =11.284 and P=0.001 the rest of the candidate SNPs in the study displayed no meaningful difference between the patient and healthy subjects.

3.3 Genotyping association: NrCAM's SNP rs10235968 showed significant association with SCZ. Comparing two genotypes CC and TT in NrCAM's SNP rs10235968, homozygote CC showed significant association with SCZ [P=0.002, df =1, OR= 4.1, 95% CI=1.651-10.18]. Comparing two genotypes CT and CC, we found significant association between homozygote CC and SCZ [P=0.001, df =1, OR= 2.96, 95% CI=1.56-5.62]. Comparing SNP rs10235968 allele positivity (CC vs. CT+TT) showed significant association between homozygote CC and SCZ [P=0.001, df =1, OR=3.175, 95% CI=1.712 - 5.887] and
comparing allele positivity (TT+CT vs. CC), once again showed significant association of homozygote CC and SCZ \([P=0.001, \text{df} = 1, \text{OR}= 3.175, 95\% \text{CI}=1.71-5.88]\). The confidence interval (CI) for odds ratio (OR) of SCZ spanned above 1. According to our results comparing heterozygote CT vs. TT showed no association with SCZ \([P=0.447, \text{df} = 1, \text{OR}=0.723, 95\% \text{CI}=0.313 - 1.670]\) and comparing allele positivity, showed no significant association between (CC+CT vs. TT) and SCZ \([P=0.08, \text{df} = 1, \text{OR}=2.033, 95\% \text{CI}=0.91 - 4.54]\). The confidence interval (CI) for odds ratio (OR) of SCZ spanned above and below 1. Genotypic association results are presented in Table 9.

Table 9: Genotypic association results

<table>
<thead>
<tr>
<th>SNP</th>
<th>Genetic model</th>
<th>OR (CI)</th>
<th>Pearson $\chi^2$ (P-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs10235968</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homozygous</td>
<td>(CC vs TT)</td>
<td>4.1(1.651 - 10.18)</td>
<td>9.767 (0.002)</td>
</tr>
<tr>
<td>Heterozygous</td>
<td>(CT vs CC)</td>
<td>2.965(1.562 - 5.627)</td>
<td>14.40 (0.001)</td>
</tr>
<tr>
<td>Heterozygous</td>
<td>(CT vs TT)</td>
<td>0.723(0.313 - 1.670)</td>
<td>0.579 (0.447)</td>
</tr>
<tr>
<td>Allele positivity</td>
<td>(CC+CT vs TT)</td>
<td>2.033(0.91 - 4.54)</td>
<td>3.075 (0.08)</td>
</tr>
<tr>
<td>Allele positivity</td>
<td>(CC vs CT+TT)</td>
<td>3.175(1.712 - 5.887)</td>
<td>11.37 (0.001)</td>
</tr>
</tbody>
</table>

SNP: Single Nucleotide Polymorphism, OR: Odds Ratio, CI: Confidence Interval, P-value: A small p-value (typically ≤ 0.05) shows strong evidence against the null hypothesis, so the null hypothesis is rejected.

3. Discussion

Frequency of all SNPs in this study showed no significant association between the candidate SNPs and SCZ except for SNP rs10235968 in NrCAM which was significantly associated with SCZ\((P=0.001)\). Decreased frequency of genotype TT and increased frequency of genotype CC for SNP rs10235968 in NrCAM were seen among patients group \((P=0.002)\). Allele-T seems to be a protective allele. SNP rs10235968, deviated from those predicted by Hardy–Weinberg equilibrium in control group \((p>0.025)\). Reduced homozygous CC/TT and increased heterozygote CT were observed among healthy group in NrCAM’s SNP rs10235968 (Table7). Probably, the heterozygote CT was increased by natural selection among healthy group and deviated from those predicted by Hardy–Weinberg. According to the 1000 genome project, the common allele for SNP rs10235968 is C with allelic frequency 0.52. The 1000 genome study was conducted only on 27 different populations and showed allele C as a common allele for SNP rs10235968 among 18 populations and allele T among at least 9 populations. The allele C frequency varies with a slight variance between different populations, thus, it may not be regarded as an ancestral allele, and more probably it is a balancing polymorphism. As a result of present study and other studies investigating association of NrCAM SNPs and schizophrenia, allele C for SNP rs10235968 suggested to be a risk allele for SCZ. The main role of SNP rs10235968 in developing of schizophrenia is still unknown. Although the candidate SNPs have been previously studied with regard to SCZ, no study has been conducted on the association of these SNPs with SCZ among the Iranian patients.
Two association researches evaluated the association of NrCAM’s SNPs with SCZ: Atz, Rollins et al. in California University reported the association of SNP rs646558 in NrCAM with the susceptibility to SCZ, and Kim, Kim et al. evaluated the association of 13 SNPs in NrCAM and SCZ among Korean population and no association was found between the examined polymorphisms and SCZ. The latter study’s results in the Korean population are not in line with those drawn from this study among the Iranian patients. SNP rs10235968 is located in the promoter of NrCAM. The role of nucleotide changes in the upstream region of NrCAM is not completely known. This region is not translated; however, since it is located in the gene’s promoter region, the epigenetic changes may affect gene translation, for instance, being methylated, is likely to cause the silencing of NrCAM (Barbeau, Liang et al. 1995). When SCZ starts to develop, expression of NrCAM is reduced due to methylation of the low CpG area (Barbeau, Liang et al. 1995). Since the expression of NrCAM in the brains of patients with SCZ strongly decreases and given the abnormal ratio of the synaptic NrCAM proteins found in the Hippocampus of schizophrenic patients (Honer, Falkai et al. 1997, Vawter, Howard et al. 1999, Vawter 2000), it can be assumed that the SNP rs10235968 carrying nucleotide C in the NrCAM’s promoter is methylated at the onset of the SCZ disorder, by way of which they affect the performance of NrCAM expression.

The cell-cell adhesion molecule, which is encoded by NrCAM gene, is vital for the formation of neurons and their axons, synaptic flexibility, myelination and highly coordinated function of the brain such as the brain’s cognitive features of memory and learning (Benson, Schnapp et al. 2000). Previous researches showed, the disruption of neuronal connection’s adhesion during neuronal cell growth in nervous system, may result in neuronal circuit dysfunction and can be the etiological foundation of many neurological disorders (Yang, Hou et al. 2014). Hargreaves, Anney et al. reported that mutations or abnormal expression of NrCAM are likely to cause alteration in synapse formation. disruption in NrCAM function, can be associated with psychiatric disorders including SCZ, Autism, Alzheimer, Mathematics learning disability and drug addiction (Sakurai 2012).

The protein NrCAM is a stimulator for division of astrocyte neuronal cell. Glucocorticoid receptor (GR) pathway contributes in NrCAM’s ability to stimulate cell division. When NrCAM proteins adhere to neuron cell, intracellular NrCAM mRNAs decrease, whereas, calreticulin mRNAs and glutamine mRNAs increase. These two genes are active in the Glucocorticoid Receptor (GR) pathway which is one of the ten signaling pathways identified as a biomarker in the Veripsych kit within blood serum of drug-Naïve schizophrenic patients (Tomasik, Schwarz et al. 2012, Sabherwal, English et al. 2016). It may be postulated that, the activity of NrCAM and the two proteins in question have an effect on each other, and a disruption in either of them will disrupt the intracellular chain of events and will cause a complication. As a result, the decreased expression of NrCAM through GR pathway can also cause disruption in development of the nervous system. NrCAM involved in CAM’s signaling pathway which has an important role in brain’s cognitive function, an attribute which is disrupted in SCZ. The association of CAM’s signaling pathway and SCZ among Chinese and European populations were previously reported (O’Dushlaine, Kenny et al. 2011,
Zhang, Yu et al. (2015) showed that the SNPs of NrCAM can affect CAM’s pathways; by way of which they can affect the brain’s cognitive function. NrCAM SNPs are likely to affect the function of protein or gene expression; through which they can affect the process of transmitting signals between neuron cells (Schmid and Maness 2008).

PRODH is one of the important known genes with regard to SCZ. The role of PRODH in the development of SCZ was frequently reported in different populations (Bassett, Marshall et al. 2008). SNP rs2238731 is situated in exon 12 of PRODH and was reported as a functional missense mutation (Bender, Almashanu et al. 2005). SNP rs2238731 is situated in a translated exon. rs2238731 (V427M) affects the function of proline dehydrogenase, the protein which is encoded by PRODH. Bender and Almashanu reported that, V427M leads to a 30-70% decrease in the activity of the proline dehydrogenase enzyme (POX) among the schizophrenic patients (Bender, Almashanu et al. 2005). The reduction in the activity of the proline dehydrogenase enzyme contributes in the development of SCZ through affecting the glutamatergic pathway and in particular, through affecting the NMDA receptors (Coyle, Tsai et al. 2003, Zinkstok, Schmitz et al. 2008). In a study carried out on relationship between polymorphisms in PRODH and brain cortical volumes, only SNP V427M (rs2238731) was found to be significantly associated with the cortical thickness. Cortical thickness in patients with genotype GA were reported to be smaller compared to those carrying genotype GG. However, this finding needs to be confirmed in a larger sample (Ota, Bellucco et al. 2014).

Variant c.562 C>T is situated in exon 3 of ANKK1. Neither schizophrenic nor non-schizophrenic people were carrying this mutated allele and no association was found between the c.562 C>T and SCZ (P=0.272). Recently, the c.562 C>T was registered by characteristic rs89721885408642 in NCBI. Variant c.7649 G>T is situated in exon 13 of ANK3. Proteins encoded by the ANK3 are those connected to cellular integral proteins. In a study conducted in Norwegian population, the role of ANK3 in susceptibility to SCZ was reported. They examined expression of ANK3 in blood and found that protein ANK3 increased in the blood of those suffering from SCZ. As a result they suggested that ANK3 mRNA might be one of the diagnostic biomarkers for diagnosing SCZ (Athanasiu, Mattingsdal et al. 2010). ANK3 is associated with other mental disorders such as autism and mental retardation (Bi, Wu et al. 2012, Iqbal, Vandeweyer et al. 2013). Increased expression of ANK3, was previously reported in blood of the patients with SCZ (Iqbal, Vandeweyer et al. 2013). The functional deficiency in protein encoded by ANK3 can be regarded as a factor for formation of psychological disorders such as mental retardation, autism and SCZ (Iqbal, Vandeweyer et al. 2013).
Conclusion: In this study for the first time, the NrCAM's polymorphisms were interrogated among a sample of Iranian patients in terms of association with SCZ. Our results suggest the association of NrCAM's SNP rs10235968 with schizophrenia disorder in an Iranian sample of patients with schizophrenia. To further understand the role of SNP rs10235968, NrCAM expression should be evaluated in future. The results obtained in this study are different from other similar studies conducted in other populations and indicate genetic diversity in different populations. NrCAM's SNPs can play important roles in the development of schizophrenia through numerous biological pathways.

Limitations: The restriction of this study was little number of sample size and low number of the polymorphisms that were evaluated in this research. In our investigation the sample size was too small to come to a decisive conclusion. Hence, further researches are suggested to support the results of this investigation. In addition, to corroborate the affiliation between NrCAM polymorphisms and SCZ among the Iranian, more polymorphisms should be evaluated.

Acknowledgment

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References


Association of SNPs with SZ


Association of SNPs with SZ


