Multiplex tetra-primer amplification refractory mutation system polymerase chain reaction to genotype SNP8NRG221533 of Neuregulin-1 gene

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Abstract
Schizophrenia is a severe neuropsychiatric disorder with symptoms such as hallucination, delusion and mental disorder. It is a complex disorder, in which genetic components play a crucial role in its pathogenesis. Among candidate genes for schizophrenia, Neuregulin 1 (NRG1) gene is the most important gene, association of which with the illness has been confirmed in several studies. Single nuclotide polymorphisms (SNPs) located 5’ upstream of NRG1 have shown significant association with schizophrenia in several populations. Here, we describe a designed simple Multiplex Tetra-Primer Amplification Refractory Mutation System - polymerase chain reaction (PCR) for genotyping single SNP (SNP8NRG221533) in the human NRG1 gene. No restriction site was found for distinguishing T and C alleles of this SNP. The developed method proved to be simple, rapid and cost effective. This technique was used to compare SNP8NRG221533 in 95 schizophrenics and 95 healthy controls. Our data demonstrate that there is a significant difference between allelic and genotypic frequencies of the two groups. These preliminary results confirm the association of the NRG1 gene with schizophrenia in an Iranian population.

Keywords: Schizophrenia; ARMS-PCR; Neuregulin1; SNP8NRG221533.

INTRODUCTION
Schizophrenia is a complex psychiatric disorder. Delusions, hallucinations, disorganized speech, grossly disorganized or catatonic behavior and negative symptoms (such as apathy, anhedonia and social withdrawal) constitute the core symptoms of schizophrenia (Norton et al., 2006).

The results of numerous family, twin, and adoption studies show conclusively that the risk of schizophrenia is increased among the relatives of affected individuals suggesting that a shared genetic factor rather than shared familial environment contributes to predisposition to schizophrenia (Green et al., 2005). Linkage analyses have identified chromosomal regions including 8p, 13q, 22q and 6p as susceptibility loci for schizophrenia (Harrison and Weinberger, 2005; Kohn and Lerer, 2002). Furthermore, a list of candidate genes for schizophrenia has been provided through relevant studies (Harrison et al., 2006; Harrison and Weinberger, 2005).

The neuregulin 1 (NRG1) gene located on 8p12.21 is one of the most promising candidates in schizophrenia genetics. Interestingly, 8p is a region that its linkage with schizophrenia has been confirmed repeatedly (Harrison et al., 2006). The NRG1 gene has more than 21 exons which could give rise to many structurally and functionally distinct isoforms, through alternative promoter usage (Harrison et al., 2006; Falls and Falls, 2003). The roles of different isoforms of NRG1 in the central nervous system have been studied extensively. The NRG1 gene encoded proteins play a wide range of
roles in the central nervous system including the modulation of neuronal migration, synaptogenesis, gliogenesis, neuron-glia communication, myelination, and neurotransmission which are completely relevant to the molecular pathology of schizophrenia. Hence, NRG1 is not only a positional candidate, but also a strong functional candidate for schizophrenia (Harrison et al., 2006; Steinthorsdottir et al., 2004; Falls and Falls, 2003; Stefansson et al., 2003).

In a previous report, Stefansson and colleagues (2002) suggested NRG1 as a candidate susceptibility gene for schizophrenia in a linkage study carried out in an Icelandic population. They found a core at-risk haplotype which is involved in the etiology of schizophrenia. This haplotype, named HapICE, was composed of five SNP markers SNP8NRG241930, SNP8NRG243177, SNP8NRG433E1006, SNP8NRG221132 and SNP8NRG221533, and two microsatellite markers 478B14-848, 420M9-1395 (Stefansson et al., 2002). Since then, HapICE and other markers adjacent to NRG1 were studied in several populations and their association with schizophrenia has been tested by several researchers. However, analyzing allele and haplotype frequencies of NRG1 in distinct populations have yielded varying results and also different alleles or haplotypes have been associated with schizophrenia (Munafo et al., 2008; Gardner et al., 2006; Munafo et al., 2006). It is also noteworthy that two studies have failed to replicate the association of NRG1 with schizophrenia in their samples. The authors concluded that the result could be due to heterogeneity of schizophrenia. Furthermore, they suggested that lack of association between schizophrenia and NRG1 in their sample could be because of a large number of loci which might be involved in pathology of schizophrenia. Allelic heterogeneity may also be contributing to the association of the NRG1 locus with schizophrenia. Finally it has been suggested that additional NRG1 association studies in other populations (Ingason et al., 2006; Thiselton et al., 2004) can be informative.

In the present study, we developed a simple rapid and cost-effective Multiplex Tetra-Primer Amplification Refractory Mutation System PCR (T-ARMS-PCR) for genotyping SNP8NRG221533 of NRG1 gene. We decided to genotype SNP8NRG221533 because it is the most commonly reported single marker of the NRG1 gene associated with schizophrenia (Thiselton et al., 2004). Notably, no restriction site could be found for distinguishing the two alleles of SNP8NRG221533. In order to test the association of SNP8NRG221533 with schizophrenia in an Iranian sample, we used T-ARMS-PCR to determine the genotypes of 95 schizophrenic patients matched with 95 healthy individuals (representing the control group).

**MATERIALS AND METHODS**

**Case-control sample:** Cases were represented by patients from Salamat and Golestan hospital of Ahwaz city (southwest of Iran). Patients were interviewed by an experienced psychiatrist and venous blood samples were collected for DNA extraction. Diagnosis were made according to the Diagnostic and Statistical Manual (DSM)-IV criteria. Control subjects were selected from the same population in the southwest of Iran. Individuals were accepted as control if they didn’t have any history of hospitalization for psychiatric disorders or history of treatment for psychiatric illness. The mean age and SD of the cases and the controls were 39.28, SD: 8.29 and 38.34, SD: 9.1, respectively. Informed consent from each patient was obtained before blood sampling. This work was approved by the Ethics Committee of Jondi Shapour University of Medical Sciences.

**DNA Extraction:** DNA was extracted from 100µl of whole blood using the DNP™ Kit (Cinnagen, Iran). Briefly, lysis solution was used to lyse blood cells, then DNA was precipitated by isopropanol selectively. Finally, the extracted DNA was washed and desalted by ethanol and dissolved in double distilled water. The quality and concentration of the extracted DNA were examined spectrophotometrically or visually after electrophoresis in 1% agarose gel.

**Polymerase chain reaction (PCR):** In order to determine the genotype of SNP8NRG221533 by multiplex PCR-ARMS, four primers were designed using the Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (Whitehead Institute, Cambridge, Massachusetts). Table 1 shows the list of primers, length of PCR products, annealing temperature and allele products. FO2215533 and RO221533 were used to amplify a 773bp fragment which was considered as a control fragment for PCR reactions. For the T allele, we expected a 302bp fragment, amplified by FT221533.
and RO221533 primers. Finally, for the C allele we expected a 522bp fragment which was the result of amplification by the RC221533 and FO221533 primers. A schematic representation of designed Tetra-Primer ARMS PCR methods has been shown in Figure 1.

In order to achieve the most suitable temperature for multiplex PCR, gradient PCR was performed. Initially, each pair of primers was optimized individually and then a Tetra-primer amplification refractory mutation system PCR (T-ARMS-PCR) was performed based on the results attained. Approximately 100 nanograms (ng) of DNA were amplified for 35 cycles using recombinant Taq polymerase (Cinnagen, Iran).

Following an initial 94°C denaturing step (5 min), samples were subjected to 35 cycles at 94°C (30 sec), 54°C (1 min), 72°C (1 min) and ending with a final extension at 72°C (5 min).

### Table1.
The sequences of primers used to genotype SNP8NRG221533 by Tetra-Primer ARMS PCR. The expected PCR products for different pair of primers are shown in the table.

<table>
<thead>
<tr>
<th>Name primer</th>
<th>Sequence</th>
<th>PCR products length</th>
<th>Allele product</th>
</tr>
</thead>
<tbody>
<tr>
<td>FO221533</td>
<td>5´ CCTGAATCTGTATAACCTTGGC 3´</td>
<td>With RO :773bp</td>
<td>Control fragment</td>
</tr>
<tr>
<td>RO221533</td>
<td>5´ GGTTTGGAATGAATATCGTCTC 3´</td>
<td>With FO :773bp</td>
<td>Control fragment</td>
</tr>
<tr>
<td>RC221533</td>
<td>5´ GGAAGCCATGTATTTTTTTATG 3´</td>
<td>With FO: 522bp</td>
<td>C allele</td>
</tr>
<tr>
<td>FT221533</td>
<td>5´ CTAAAAAAGGATATATGATTTTTG 3´</td>
<td>With RO: 302bp</td>
<td>T allele</td>
</tr>
</tbody>
</table>

### Statistical Analysis:
For the purpose of statistical analysis, the association of SNP8NRG221533 with schizophrenia as a single marker was examined by using the Chi-square test to compare frequency differences of the SNP8NRG221533 alleles and genotypes between the groups studied. A conventional $p$-value of $\leq 0.05$ was considered significant.

### RESULTS

In this study, a simple Tetra-primer ARMS-PCR was used for genotyping the SNP8NRG221533 of the NRG1 gene. We distinguished different genotypes of individuals using this method (Fig. 2). The accuracy of the method was confirmed by sequencing some of the samples. The genotypes determined by sequencing...
(Macrogen, Korea) were consistent with genotypes determined by Tetra-Primer ARMS PCR (Fig. 3).

Subsequently, this method was applied for genotyping SNP8NRG221533 among 95 schizophrenics matched with 95 healthy individuals. After determining the genotypes of both groups, the Hardy-Weinberg equilibrium was tested in the case and control populations. Statistical analyses showed that genotype frequencies were in Hardy-Weinberg equilibrium in the controls ($\chi^2=0.12$, df=2, $p \leq 0.05$) and patients ($\chi^2=0.070$, df=2, $p \leq 0.05$). The genotype frequencies in the patients’ group were as follows: CC 36%; CT 57%; TT 7% vs. the genotype frequencies in the control group: CC 16%; CT 76%; TT 8%.

The C allele of SNP8NRG221533 is the risk allele which has been associated with schizophrenia in other populations. The frequency of C allele in patients was 64.2 while this number in the control group was 53.68. Therefore, the frequency of the C-allele in the patient group was 1.19 fold higher compared with the control subjects, demonstrating significant difference in allelic frequency ($\chi^2=4.35$, df = 1, $p \leq 0.037$).

As the genotypic distribution between the two groups was compared, we found that the homozygocity of C allele is associated with a higher risk of schizophrenia. Our analyses show that there is a significant difference between the CC genotype versus CT+TT genotypes in the two groups ($\chi^2=9.6$, df = 1, $p \leq 0.002$). Table 2 shows the summarized results of statistical analyses.

### DISCUSSION

Many methods, such as single-strand conformation polymorphism analysis and DNA HPLC (dHPLC), are suitable for genotyping SNPs. These methods, however, require specialized equipments and most importantly, are not designed for screening of known SNPs in a large number of samples. Although the application of restriction enzymes for genotyping is a straightforward and suitable method, it is not applicable for all SNPs because some SNPs do not have a restriction site by which different alleles could be distinguished.

In this study, we developed a simple tetra-primer amplification refractory mutation system PCR (T-ARMS-PCR) for screening the SNP8NRG221533 of the NRG1 gene. Conventional ARMS-PCR amplifies the two alleles in two different PCR reactions (Old et al., 1990; Newton et al., 1989). In contrast, T-ARMS-PCR

### Table 2. Allelic and genotypic frequencies for SNP8NRG221533 in cases and controls.

<table>
<thead>
<tr>
<th>SNP Name</th>
<th>Subject (number)</th>
<th>C allele frequency (%)</th>
<th>Genotypic frequency (%)</th>
<th>(C) allele vs. (T)</th>
<th>CC vs. CT+TT genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNP8NRG21533</td>
<td>Case (95)</td>
<td>64.2</td>
<td>34(0.36) 54(0.57) 7(0.07)</td>
<td>$p \leq 0.035$</td>
<td>1 4.35 $\leq 0.002$ 1 9.6</td>
</tr>
<tr>
<td></td>
<td>Control (95)</td>
<td>53.68</td>
<td>15(0.16) 72(0.76) 8(0.08)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

df: Degree of Freedom, $p$: P-value, $\chi^2$: Chi square.
PCR amplifies both alleles, together with a control fragment in a single tube PCR reaction (Ye et al., 2001). The designed T-ARMS-PCR technique proved to be simple, rapid and does not require a special instrument for genotyping known SNPs.

Using this method, we tested the association of single NRG1 polymorphisms with schizophrenia in a sample population from Iran. The association of SNP8NRG221533 with schizophrenia has been confirmed in other populations repeatedly (Harrison et al., 2006; Tosato et al., 2005). However, attempts to replicate the associated studies are of great value and have been proposed as guidelines to avoid spurious results. Importantly, replication of an association study in a new population can lead to generation of invaluable data regarding the role of a gene in the pathogenesis of that disease (Cardon and Bell, 2001). It is worth mentioning that the association of NRG1 with schizophrenia in a demographically distinct population would be compelling evidence in favor of true association between NRG1 and schizophrenia.

Previous reports on SNP8NRG221533 indicated that the risk allele (C) frequency ranged from 29.6 to 59.2% across different populations, all studies showed a significant P-value of 0.0065 (Li et al., 2006). In comparison with original study, the frequency of C allele in our samples was higher than the Icelandic population (64.2 versus 36.4) (Stefansson et al., 2003; Stefansson et al., 2002). This variation in allele frequency is due to the different linkage disequilibrium pattern between populations with different ancestry. It is noteworthy that the frequency of this allele in our samples showed more similarity to that of the Asian population than to European samples (Fukui et al., 2006; Li et al., 2006; Munafò et al., 2006; Petroshen et al., 2005; Zhao et al., 2004; Yang et al., 2003).

The NRG1 gene has more than 20 known exons and SNP8NRG221533 is located upstream of the first exon (Steinthorsdottir et al., 2004). The region mainly controls the expression of glial growth factor 2 (GGF2), the deficiency of which has been thought to play an important role in the pathology of schizophrenia (Steinthorsdottir et al., 2004; Pulver et al., 2000). Therefore, the results of this study add weight to the idea that there could be some functional variants in this region which play a crucial role in the pathogenesis of schizophrenia.

In summary, the designed Tetra-Primer ARMS-PCR provides a rapid, reproducible, and cost-effective method for genotyping the SNP8NRG221533 of the NRG1 gene. Also, this study supports the view that NRG1 can be considered as a candidate gene for Schizophrenia in the Iranian population. Nevertheless, further studies are needed to confirm the exact association of NRG1 gene with schizophrenia in the Iranian population.

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References


