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 schizophernics 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.

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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

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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, Steafansson 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 Hap_{ICE} , 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, Hap_{ICE} 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

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.

Name primer	Sequence	PCR products length	Allele product
FO221533	5′ CCTGAACTCTGTATAACATTGGC 3′	With RO :773bp	Control fragment
RO221533	5′ GGTTTGGAATGATAAGCCTCTCAC 3′	With FO :773bp	Control fragment
RC221533	5′ GGAAGCCATGTATCTTTATTTTG 3′	With FO:522bp	C allele
FT221533	5′ CTAAAAAAGAGATATATGATATTTGGT 3′	With RO: 302bp	T allele

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

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 ≤ 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
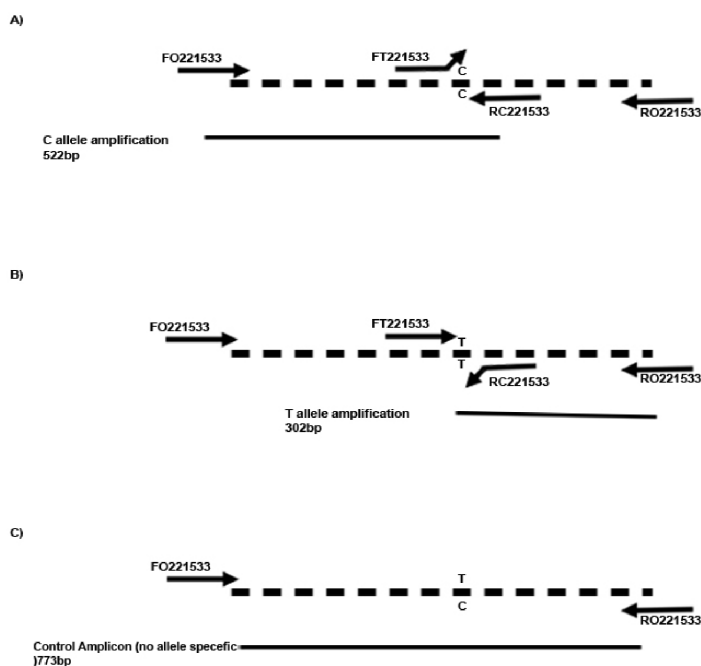

Figure 1. Schematic representation of the designed Tetra Primers ARMS-PCR. A) The specific amplification of the 522 bp fragment which represents the C allele. B) The specific amplification of the 304 bp fragment which represents the T allele. C) Amplification of a 775 bp control fragment which is not specific for any allele.
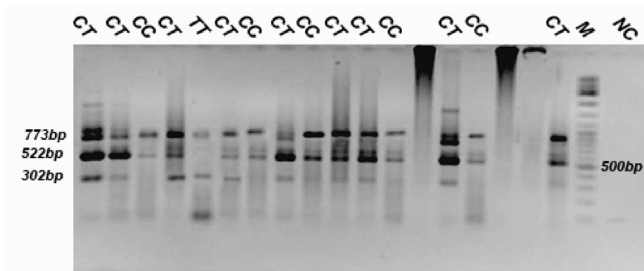
Figure 2. The results of used Tetra-Primers-ARMS PCR for genotyping of SNP8NRG221533. The 773 bp band represents the control fragment which is always amplified by FO221533 and RO221533 regardless of genotype of samples. The 522bp fragment amplification indicates the presence of C allele and amplification of 302 bp fragment shows the presence of T allele at SNP site. The genotype of each sample is shown on the top.
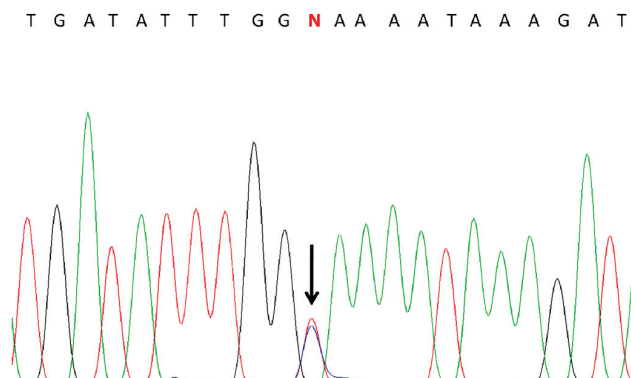
T G A T A T T T G G **N** A A A A T A A A G A T

Figure 3. The results of sequencing were consistent with determined genotype by T-ARMS-PCR. This picture shows a heterozygote sample for SNP8NRG221533. The presence of two peaks at SNP site indicates the hetrozygosity of sample.

(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 (χ^2=0.12, df=2, $p \leq 0.05$) and patients (χ^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 (χ^2=4.35, df = 1, $p \leq 0.037$).

As the genotypic distrubution 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 (χ^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-

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

SNP Name	Subject (number)	C allele frequency (%)	Genotypic frequency (%)			(C) allele vs. (T)			CC vs. CT+TT genotypes		
			CC	CT	TT	p	df	χ^2	p	df	χ^2
SNP8NRG2 21533	Case (95)	64.2	34(0.36)	54(0.57)	7(0.07)	≤ 0.035	1	4.35	≤ 0.002	1	9.6
	Control (95)	53.68	15(0.16)	72(0.76)	8(0.08)						

df: Degree of Freedom, P: P-value, χ^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 techniqueproved 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; Munafo *et al.*, 2006; Petryshen *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.

Acknowledgements

References

Cardon LR, Bell JI (2001). Association study designs for complex diseases. *Nat Rev Genet*. 2: 91-99.

Falls DL, Falls DL (2003). Neuregulins: functions, forms, and signaling strategies Neuregulins and the neuromuscular system: 10 years of answers and questions. *Exp Cell Res*. 284: 14-30.

Fukui N, Muratake T, Kaneko N, Amagane H, Someya T (2006). Supportive evidence for neuregulin 1 as a susceptibility gene for schizophrenia in a Japanese population. *Neurosci Lett*. 396: 117-120.

Gardner M, Gonzalez-Neira A, Lao O, Calafell F, Bertranpetit J, Comas D (2006). Extreme population differences across Neuregulin 1 gene, with implications for association studies. *Mol Psychiatry*. 11: 66-75.

Green EK, Raybould R, Macgregor S, Gordon-Smith K, Heron J, Hyde S, Grozeva D, Hamshere M, Williams N, Owen MJ, O'Donovan MC, Jones L, Jones I, Kirov G, Craddock N (2005). Operation of the schizophrenia susceptibility gene, neuregulin 1, across traditional diagnostic boundaries to increase risk for bipolar disorder. *Arch Gen Psychiatry*. 62: 642-648.

Harrison PJ, Law AJ, Law AJ, Lipska BK, Weickert CS, Hyde TM, Straub RE, Hashimoto R, Harrison PJ, Kleinman JE, Weinberger DR (2006). Neuregulin 1 and schizophrenia: genetics, gene expression, and neurobiology Neuregulin 1 transcripts are differentially expressed in schizophrenia and regulated by 5' SNPs associated with the disease. *Biol Psychiatry*. 60: 132-140.

Harrison PJ, Weinberger DR (2005). Schizophrenia genes, gene expression, and neuropathology: on the matter of their convergence. *Mol Psychiatry*. 10: 40-68; image 45.

Ingason A, Soeby K, Timm S, Wang AG, Jakobsen KD, Fink-Jensen A, Hemmingsen R, Berg Rasmussen H, Werge T (2006). No significant association of the 5' end of neuregulin 1 and schizophrenia in a large Danish sample. *Schizophr Res*. 83: 1-5.

Kohn Y, Lerer B (2002). Genetics of schizophrenia: a review of linkage findings. *Isr J Psychiatry Relat Sci*. 39: 340-351.

Li D, Collier DA, He L (2006). Meta-analysis shows strong positive association of the neuregulin 1 (NRG1) gene with schizophrenia. *Hum Mol Genet*. 15: 1995-2002.

Munafo MR, Attwood AS, Flint J (2008). Neuregulin 1 genotype and schizophrenia. *Schizophr Bull* 34: 9-12.

Munafo MR, Thiselton DL, Clark TG Flint J (2006). Association of the NRG1 gene and schizophrenia: a meta-analysis. *Mol Psychiatry*. 11: 539-546.

Newton CR, Graham A, Heptinstall LE, Powell SJ, Summers C, Kalsheker N, Smith JC, Markham AF (1989). Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). *Nucleic Acids Res*. 17: 2503-2516.

Norton N, Williams HJ, Owen MJ (2006). An update on the genetics of schizophrenia. *Curr Opin Psychiatry*. 19: 158-164.

Old JM, Varawalla NY, Weatherall DJ (1990). Rapid detection and prenatal diagnosis of beta-thalassaemia: studies in Indian and Cypriot populations in the UK. *Lancet* 336: 834-837.

Petryshen TL, Middleton FA, Kirby A, Aldinger KA, Purcell S, Tahl AR, Morley CP, McGann L, Gentile KL, Rockwell GN, Medeiros HM, Carvalho C, Macedo A, Dourado A, Valente J, Ferreira CP, Patterson NJ, Azevedo MH, Daly MJ, Pato CN, Pato MT, Sklar P (2005). Support for involvement of neuregulin 1 in schizophrenia pathophysiology. *Mol Psychiatry*. 10: 366-374.

Pulver AE, Mulle J, Nestadt G, Swartz KL, Blouin JL, Dombroski B, Liang KY, Housman DE, Kazazian HH, Antonarakis SE, Lasseter VK, Wolyniec PS, Thornquist MH, McGrath JA (2000). Genetic heterogeneity in schizophrenia: stratification of genome scan data using co-segregating related phenotypes. *Mol Psychiatry*. 5: 650-653.

Stefansson H, Sarginson J, Kong A, Yates P, Steinthorsdottir V, Gudfinnsson E, Gunnarsdottir S, Walker N, Petursson H, Crombie C, Ingason A, Gulcher JR, Stefansson K, St Clair D (2003). Association of neuregulin 1 with schizophrenia confirmed in a Scottish population. *Am J Hum Genet*. 72: 83-87.

Stefansson H, Sigurdsson E, Steinthorsdottir V, Bjornsdottir S, Sigmundsson T, Ghosh S, Brynjolfsson J, Gunnarsdottir S, Ivarsson O, Chou TT, Hjaltason O, *et al.* (2002). Neuregulin 1 and susceptibility to schizophrenia Association of neuregulin 1 with schizophrenia confirmed in a Scottish population. *Am J Hum Genet*. 71: 877-892.

Steinthorsdottir V, Stefansson H, Ghosh S, Birgisdottir B, Bjornsdottir S, Fasquel AC, Olafsson O, Stefansson K, Gulcher JR (2004). Multiple novel transcription initiation sites for NRG1. *Gene* 342: 97-105.

Thiselton DL, Webb BT, Neale BM, Ribble RC, O'Neill FA, Walsh D, Riley BP, Kendler KS (2004). No evidence for linkage or association of neuregulin-1 (NRG1) with disease in the Irish study of high-density schizophrenia families (ISHDSF). *Mol Psychiatry*. 9: 777-783.

Tosato S, Dazzan P, Collier D (2005). Association between the neuregulin 1 gene and schizophrenia: a systematic review. *Schizophr Bull*. 31: 613-617.

Yang JZ, Si TM, Ruan Y, Ling YS, Han YH, Wang XL, Zhou M, Zhang HY, Kong QM, Liu C, Zhang DR, Yu YQ, Liu SZ, Ju GZ, Shu L, Ma DL Zhang D (2003). Association study of neuregulin 1 gene with schizophrenia. *Mol Psychiatry*. 8: 706-709.

Ye S, Dhillon S, Ke X, Collins AR, Day IN (2001). An efficient procedure for genotyping single nucleotide polymorphisms. *Nucleic Acids Res*. 29: E88-88.

Zhao X, Shi Y, Tang J, Tang R, Yu L, Gu N, Feng G, Zhu S, Liu H, Xing Y, Zhao S, Sang H, Guan Y, St Clair D, He L (2004). A case control and family based association study of the neuregulin1 gene and schizophrenia. *J Med Genet*. 41: 31-34.