RESEARCH ARTICLE

Association between a single nucleotide polymorphism in neuregulin-1 and schizophrenia in Pakistani patients

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Abstract

Objective: To determine the association of single-nucleotide polymorphism8nrg433E1006 in the neuregulin-1 gene associated with schizophrenia.

Methods: This case-control study was conducted at the Fountain House, Lahore, and the psychiatric clinics at the Aga Khan University, Karachi, from 2010 to 2013.The total genomic deoxyribonucleic acid was isolated and single-nucleotide polymorphism8nrg433E1006 was screened by nested polymerase chain reaction followed by sequencing. These sequences, from patients and controls, were aligned with the human neuregulin-1-glial growth factor 2 gene sequence, which served as a reference sequence. The single nucleotide polymorphism genetic algorithm was characterised at position 433 in the neuregulin-1 gene by aligning test and control sequences with the neuregulin-1-glial growth factor 2reference sequence using ClustalW algorithm, implemented in the BioEdit software.

Results: Of the 630 samples, 321(51%) were of cases and 309(49%)of controls. Moreover, 99(30.8%) cases and 79(25.6%) controls rendered correct neuregulin-1 gene frames. Of them, the single-nucleotide polymorphism8nrg433E1006 was present in 62(62.6%) cases and 24(30.4%) controls. The analysis showed that the odds ratio of having schizophrenia is 3.8 times higher in the presence of this single-nucleotide polymorphism at the 92 bp of neuregulin-1 gene with the 95% confidence interval(p=0.0001).

Conclusion: There was a strong association of single-nucleotide polymorphism8nrg433E1006 in the neuregulin-1 gene with schizophrenia.

Keywords: Schizophrenia, NRG-1 gene, SNP8nrg433E1006. (JPMA 68: 747; 2018)

Introduction

Schizophrenia is a mental disorder which runs a chronic course leading to brain dysfunction and the deterioration of personality.[1] It affects one individual out of a hundred. Heritability and genetic risk is postulated to play a major role in the aetiology of schizophrenia.[2] The completion of the human genome project has allowed us to understand the molecular basis of a number of human diseases. Research pertaining to genetics of schizophrenia has also progressed rapidly over the past decade. High throughput mapping and scanning of the human genome by emerging technologies has enabled us to identify disease-specific gene mutations quickly. Genetic mapping follows the descent down the generations of deoxyribonucleic acid (DNA) markers in pedigree and their segregation with illness. Either a very large single pedigree or a collection of smaller families where the effects of linkage is additive is studied. The rates of schizophrenia were greater among the biological relatives of the schizophrenic adoptees than among the relatives of controls; a finding that supports the genetic hypothesis. Furthermore, the rate of schizophrenia was not increased among couples that adopted the schizophrenic adoptees, suggesting that environmental factors were not of substantial importance.

Neuregulin-1 (NRG-1) is a gene that has shown perhaps one of the most robust linkages in terms of disease causation.[3] NRG-1 is located on chromosome 8p and is a large gene of about 1.2 Mbp, with at least 30 exons and nine potential promoters. Chromosome 8 has been one of the more problematic chromosomes in terms of sequence assembly and marker orders, with further complications arising due to inversion polymorphisms[4] and deletions.[5] This may, in part, explain the differences in the intervals reported. Alternatively, there may be a second schizophrenia gene on chromosome 8p. A core haplotype (HAP ice) was identified by Stefansson et al. in the original study on the Icelandic population that first implicated this gene. Recently, the same region was identified in the Irish, Scottish and Chinese populations. NRG-1 is a plausible susceptibility gene as it plays a direct role in the expression of the N-methyl-D-aspartate (NMDA) receptor by regulating of glutamate and the neurotransmitters. The

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current study was planned to determine if single nucleotide polymorphism in the NRG-1 has an association with schizophrenia. To the best of our knowledge, no previous study has explored the association of NRG-1 mutation with schizophrenia in a Pakistani population. Unravelling the genetic risk associated with schizophrenia has the potential to stimulate further research in the area of drug-designing to treat this serious mental disorder.

Patients and Methods

This case-control study was conducted at the Fountain House, Lahore, and the psychiatric clinics at the Aga Khan University, Karachi, from 2010 to 2013. The sample size required for this study was estimated based on the prevalence of exposure to risk factors among controls (%), and the number of cases required to detect a relative risk (RR) of 1.5. In an earlier study exploring the same issue (a five-markerhaplotype) in Han Chinese population, the risk was estimated to be 27% among the controls and 37% in cases.[6] The sample size was calculated using the following formula:

$$N = (Z_1-\alpha/2 + Z_1-\beta)^2 (p_1(1 - p1) + p_2(1 - p_2))/ (p_1 - p_2)^2$$

The proportion in the control group was taken as null hypothesis while the proportion in cases was taken as the alternative hypothesis.[7] In the above equation, p_1 representeds group one and p_2 represented group 2. With the power of 80%, and significance level of 5%, the minimum estimated sample size was 300 cases and 300 controls.

Based on the afore-mentioned calculations, patients with the clinical diagnosis for schizophrenia, as per the 10th revision of the International Statistical Classification of Diseases and Related Health Problems(ICD 10),[8] were included in the study. The individuals with comorbid substance use disorder, mental retardation, mood disorder, head injury and schizoaffective disorder were excluded. Sampling was carried out on patients whose relatives gave consent. The patients were assessed for clinical remission, social and occupational functioning. Details of socio-demographic variables were collected on a predesigned proforma. All controls and cases consented to participate in the study. Information on the educational status, marital status, occupation, family history of psychiatric disorder, birth order was collected. First degree relatives were enrolled as controls.

Then, 5ml blood was collected from the enrolled patients and the controls. DNA extracted from the blood samples was performed using a genomic DNA isolation kit (Qiagen) as given in the manufacturer's protocol. The isolated DNA was stored at -20 degrees Celsius.

DNA isolated from the blood samples of cases and controls was used to amplify single-nucleotide polymorphism (SNP) 8nrg433E1006 through polymerase chain reaction (PCR), which lies in the 5' exon of intravenous glial growth factor 2 (GGF2), using nested PCR strategy, followed by sequencing. The first round was performed using primers CCTACCCCTGCACCCCCTAAATAA and CTTCCTGTCGACTGCCCCCTGCT. In the first round, 30ng of genomic DNA was amplified in the presence of 3.5pmol of each primer, 0.25U Taq polymerase, 0.2mM deoxynucleotide triphosphates (dNTPs), 10% dimethyl sulfoxide (DMSO), 1% glycerol and 2.5mM magnesium chloride (MgCl). Cycling conditions for the first round were: 95°C for 10 minutes, followed by 40 cycles of 94°C for 15 seconds, annealing at 63°C for 30 seconds, and extension at 72°C for 1 minute. The second round was performed using the same concentration of inner primers, TGCCACTACTGCTGCTGCT and ACCTTTCCCTCGATCACCAC. Except for the addition of 3ul of the first amplification reaction, as a template, to 27ul of the mixture, conditions were the same as in the first amplification reaction. Cycling conditions for the second amplification were 95°C for 10 minutes, followed by 35 cycles at 94°C for 15 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 1 minute. The 163 bp PCR product was sent for sequencing to Macrogen with inner forward primer.

The NRG1 gene sequences from patients and controls were aligned with human NRG1-GGF2 gene sequence (accession number NM_013962.2), which served as a reference sequence. The SNP (G/A) was characterised at position 433 in NRG1 gene. The position 433, after aligning the NRG1-GGF2 gene, corresponded to position 92 in the alignment. We used NRG1-GGF2 sequence (position 92 in alignments) to locate SNP in the test and control groups. The test and control sequences were aligned with NRG1-GGF2 sequence using ClustalW algorithm implemented in the BioEdit software, and SNPs at position 92, in the respective test and control sequences were identified, using position 92 of the NRG1-GGF2 gene as reference. The statistical association between SNP(s) in NRG-1 with cases and controls was assessed by calculating odds ratio. The study was given ethical approval by the institutional ethics review committee and was done in compliance with ethical standards highlighted in the Declaration of Helsinki.

Results

Of the 630 samples, 321(51%) were of cases and 309(49%) of controls. Overall, 261(41.4%) cases and 252(40%) controls were recruited from the Lahore centre and 60(9.5%) cases and 57(9%) controls from the Karachi

Table-1: Socio-demographic Characteristics: The socio-demographic characteristics of both cases and controls are shown.

Variables	Cases N=321(%)	Controls N=305(%)
Age (In Years)		
< 10	-	-
20-Nov	6(1.9)	14(4.6)
21-30	62(19.4)	119(39)
31-40	138(43.1)	95(31.1)
41-50	87(27.2)	55(18.0)
51-60	24(7.5)	17(5.6)
61-70	3(0.9)	5(1.6)
Gender		
Male	192(59.8)	238(78.5)
Female	129(40.2)	65(21.5)
Education		
Illiterate	32(10)	20(6.6)
Primary	60(18.8)	55(18.1)
Secondary	117(36.7)	72(23.7)
Intermediate	70(21.9)	95(31.3)
Graduate	32(10)	47(15.5)
Postgraduate	8(2.5)	15(4.9)
Marital Status		
Single	223(69.5)	76(24.9)
Married	44(13.7)	225(73.8)
Divorced	39(12.1)	1(0.3)
Widowed/er	1(0.3)	3(1.0)
Separated	14(4.4)	-
Occupation		
Unemployed	267(83.4)	1(0.3)
Businessmen	5(1.6)	11(3.6)
Housewife	8(2.5)	13(4.3)
Professional	13(4.1)	50(16.4)
Skilled labourer	8(2.5)	120(39.5)
Unskilled labourer	1(0.3)	89(29.3)
Students	15(4.7)	14(4.6)
Others	3(1.0)	6(1.8)
Religion		
Islam	319(99.4)	259(84.9)
Christian	1(0.3)	43(14.1)
Hindu	1(0.3)	3(1.0)
Family History of Mental Health		
Yes	154(48.1)	-
No	81(25.3)	-
Don't know	85(26.6)	3(1.0)
Past Contact		
Psychiatrist	164(51.6)	-
General practitioner	97(30.5)	-
Hakim	21(6.6)	-
Faith healer	33(10.4)	-
Other	3(0.9)	1(0.3)
No Psychiatric visit	-	49(16.5)
Not answer	-	253(82.9)
Family History of Schizophrenia		
Yes	103(32.2)	16(5.2)
No	89(27.8)	33(10.1)
Don't know	128(40.0)	3(1.0)
	-	253(82.9)
Birth Order		
1st	40(12.5)	13(28.9)
2nd	90(28.1)	9(20)
3rd	75(23.4)	7(15.6)
4th	41(12.8)	8(17.8)
5th	25(7.8)	2(4.4)
6th	9(2.8)	3(6.7)
7th	3(0.9)	-
8th	-	1(2.2)
10th	-	1(2.2)
11th	-	1(2.2)
Don't know	37(11.6)	-

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Table-2: The frequency of SNP SNP8nrg433E1006in NRG-1 gene in cases and control: The single SNP G/A was characterised at position 433 in NRG1 gene. The position 433, after aligning the NRG1-GGF2 gene reference sequence, corresponded to position 92 in the alignment.

	Guanine (%)	Adenine (%)	Thymine (%)	Cytosine (%)	Total (%)
Samples (n=99)	62(62%)	53(53%)	2(2%)	2(%)	99
Controls (n=79)	24(30%)	23(30%)	14(17)	9(12%)	79

SNP: Single-nucleotide polymorphism
NRG1: Neuregulin-1
G/A: Genetic algorithm
GGF2: Glial growth factor 2.

centre. Thus, an ethnically diverse sample was recruited as planned. Moreover, there were 418(66.3%) males (181(43%) cases; 237(57%) controls) and 212(33.7%) females (140(66%) cases; 72(34%) controls). Besides, 117(36.4%) cases and 72(23.3%) controls had secondary school education; 44(13.7%) cases and 225(72.8%) controls were married; and 267(83%) cases were unemployed and dependent on family/others for source of financial support. Family history of definite mental illness was reported in 154(48%) cases, while family history of schizophrenia was reported in 103(32%) cases (Table-1).

The total genomic DNA was isolated and SNP8nrg433E1006 was screened by nested PCR followed by sequencing. It was found that 99(30.8%) and 79(25.6%) sequences from cases and controls, respectively, showed correct NRG1 gene frames (Table-2).

Of them, the nucleotide G was present in 62(62.6%) sequences from cases, while it was present in 24(30.4%) sequences from subject group. The analysis showed that the odds ratio (OR) of having schizophrenia is 3.84 times higher in the presence of this SNP at the 92 bp of NRG-1 gene with the 95% confidence interval (CI), 2.0471 to

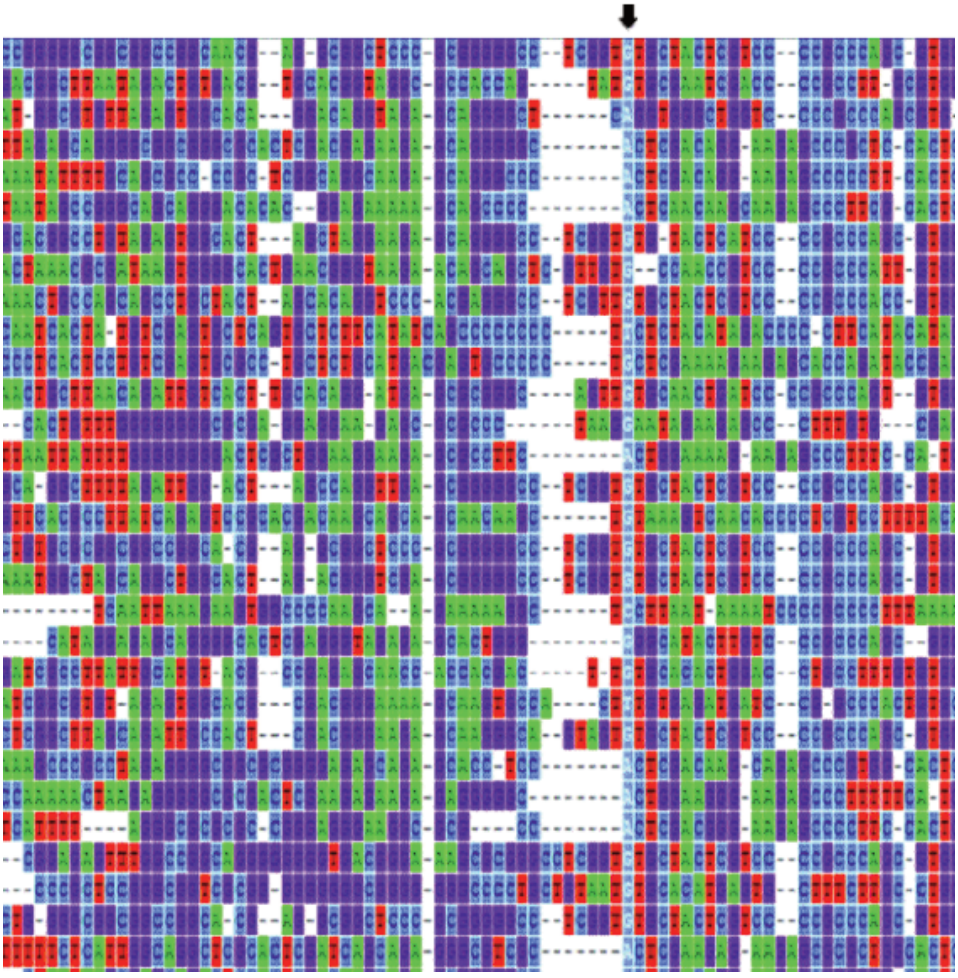
Table-3: Odds ratio of SNP at NRG-1 mutation between cases and controls. Odds ratio was found to be 3.84, 95% C.I, 2.0471 to 7.2033, P-value < 0.0001.

	Guanine Nucleotide	Non-guanine Nucleotide	Total
Cases	62(62%)	57 (55%)	119
Controls	24 (30%)	46 (59%)	70
	86	103	189

SNP: Single-nucleotide polymorphism
NRG1: Neuregulin-1.

7.2033, and highly significant difference (p=0.0001) (Table-3).

We used NRG1-GGF2 sequence (position 92 in alignments) to locate SNP in the test and control groups. The SNP was identified to be located at the 92ndbp of the 163bp-long amplified product (Figure).



GGF2: Glial growth factor 2

Figure: NRG-1 Gene Sequence alignment: The Neuregulin 1 (NRG1) gene sequences from patients and controls were aligned with Human NRG1-GGF2 gene sequence (Accession number NM_013962.2), which served as a reference sequence. The single-nucleotide polymorphism (SNP) G/A has been characterized at position 433 in NRG1 gene. The position 433, after aligning the NRG1-GGF2 gene, corresponded to position 92 in our alignment (Black arrow), and the same position was used to detect the SNP in the respective test and control sequences.

Discussion

The NRG1gene is identified as a susceptibility gene for schizophrenia by using association methodologies based on microsatellite markers, detecting SNPs. In the present case-control study, we investigated the frequency of at-risk gene markers in a Pakistani population. One of the roles that NRG1 plays in the adult central nervous system (CNS) is the expression and phosphorylation of certain neurotransmitter-receptor subunits, in certain neurons and their related complexes, in an activity dependent manner.[9-12] NRG1 does not appear to be restricted to a single neurotransmitter system; rather, it appears to play a role in NMDA and acetylcholine, as well as g-amino butyric acid-receptor, at least in certain neuronal systems, the prime function of this is to regulate the composition of neurotransmitter receptors in maturing synapses in the brain.[9,11-13]

With the completion of the Human Genome Project in April 2003, public gene databases have led to genome wide association studies that have identified many candidate genes. However, these findings have not always been replicated in different studies and merely signify an association with schizophrenia. Two recent meta-analyses have supported possible linkage at 1q, 2q, 3p, 5q, 6p, 8p, 11q, 13q, 14p, 20q and 22q. Genes such as NRG1 and catechol O-methyltransferase (COMT) have been identified as candidates for causing schizophrenia. Epigenetic influences such as increased methylation on dopamine receptor D2 (DRD2) gene in normal individuals has also been proposed which is not found in monozygotic twin with schizophrenia. Other genes proposed in 2005 by Harrison and Weinberger included G72, D-amino acid

oxidase (DAO), regulator of G-protein signalling 4 (RGS4), proline dehydrogenase (PRODH), mGluR3, disrupted in schizophrenia 1 (DISC1), COMT, NRG1 and dystrobrevin-binding protein 1(dysbindin, or DTNBP1).

COMT is the most plausible of the susceptibility genes. Located on chromosome 22q11, it codes for the post-synaptic intracellular enzyme COMT, which is involved in the methylation and degradation of the catecholamine neurotransmitters dopamine, epinephrine, and norepinephrine. The gene has multiple variants that have an effect on its activity. A variant with the valine-methionine configuration is known to have a protective effect against schizophrenia as compared to the valine-valine variant which has a greater dopamine degradation effect.

Another gene that codes for the nitric oxide synthase 1 adaptor protein (NOS1AP) is found in high concentration in inhibitory neurons in the brain. Nitric oxide regulates intracellular enzyme activities and hence works as a messenger. Using a recently developed statistical technique of linkage disequilibrium, researchers have succeeded in identifying a single-nucleotide polymorphism (SNP) associated with increased levels of expression of this gene in post-mortem brain samples from patients with schizophrenia.

Another gene associated with schizophrenia is RELN, which codes for the protein reelin that is involved in the regulation of brain development and GABAergic activity. In an international study, a common variant in this gene increased the risk of developing schizophrenia, but only in the female population.

Numerous studies have also found evidence linking abnormalities in neurodevelopmental genes with an increased susceptibility to schizophrenia. Genes such as DISC1, NRG1and DTNBP1 have been associated with schizophrenia in a number of studies, but with a variability of results. Such findings are also indicative of the hypothesis that multiple genetic variations may result in a common clinical outcome in the case of schizophrenia. A case-control study conducted through the collaborative efforts of researchers from University of North Carolina School of Medicine, the Karolinska Institute in Sweden, the Stanley Centre for Psychiatric Research at the Broad Institute of MIT and Harvard, and the Mt. Sinai School of Medicine in New York published in 2013 concluded that two genetically determined processes were of particular importance to schizophrenia, the calcium channel pathway and the micro-ribonucleic acid (micro-RNA) 137 pathway.

The identification of numerous susceptible genes establishes the basis of having a first-degree relative with schizophrenia being a major risk factor for developing the disorder. Since the genetic make-up of twins is almost identical, the expected prevalence in susceptible pairs amongst them would be high. Studies have reported a higher concordance rate amongst monozygotic twins (MZ) (43%) as compared to dizygotic twins (22%).[14] The indistinguishable genetic basis of MZ twins explains the association. However, there is substantial discordance within the monozygotic pairs which highlights the importance of non-genetic factors. In accordance with the two-hit hypothesis of schizophrenia, a combination of genetic susceptibility combined with a distinct developmental insult can ultimately lead to the occurrence of a full clinical syndrome.[15] Proposed mechanisms for discordance amongst MZ twins are environmental factors and other epigenetic processes which regulate gene expression via DNA, histone proteins and chromatin modifications. Furthermore, significant differences in DNA methylation of peripheral tissue samples between MZ twins explain the basis of phenotypic variations amongst them. The differentially methylated positions, cg23933044, located in the promoter regulatory region of C5ORF42, revealed that affected twin had reduced DNA methylation as opposed to their unaffected co-twin.[16] Thus, significant associations can be formed between MZ twins and schizophrenia based on genetic, epigenetic and environmental factors.

Despite investment of many years of research, the aetiology of schizophrenia has yet to be unravelled. The methods used for mapping susceptibility genes, including genome-wide association studies (GWAS) and copy number variation (CNV) studies, have progressed over the years. Even so, some patients with schizophrenia have no known family history of the disorder and this may be the result of new mutations. Support for this phenomenon lies in the observation that de novo mutations are more common in patients with schizophrenia as compared to the normal population.

The major strengths of this study were that it was carried out on a large number of subjects from two different cities of Pakistan. Additionally, by employing sequencing strategy, we were able to carry out in-depth genetic analysis of NRG-1 gene, which would not have been possible by simple visualisation of SNP-specific band on agarose gel. The limitations are that although our sample was derived from genetically diverse population in Karachi and Lahore, this cannot be assumed to be representative of the population at large.

Conclusion

An SNP was identified to be located at the 92ndbp of the 163bp-long amplified product. Future studies exploring similar associations, stratified at various sites, would add further evidence to the body of literature in this important public health concern.

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Conflict of Interest: None.

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