METHODOLOGY
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>DSM</td>
<td>Diagnostic and Statistical Manual of Mental Disorders</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene Diamine Tetra Acetic acid</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>ds DNA</td>
<td>double stranded DNA</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>Tm</td>
<td>Annealing Temperature</td>
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<tr>
<td>DNTP</td>
<td>Di Nucleotide Triphosphate</td>
</tr>
<tr>
<td>SCZ</td>
<td>Schizophrenia</td>
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</tbody>
</table>
MATERIAL REQUIRED

Agarose

Ammounium Chloride NH₄Cl

Blood

Chloroform CHCl₃

DNA

Ethanol C₂H₅OH

Ethylene Diamine EDTA
Tetra acetic acid

Magnesium Chloride MgCl₂

Oligonucleotides

Phenol C₆H₅OH

Potassium Chloride KCl

Proteinase-K

Tris- Ethylene Diamine Tris EDTA
Tetra acetic acid

Tris Hydrochloric acid Tris-HCl

Sodium Acetate CH₃COONa

Sodium Chloride NaCl

Sodium Hydroxide NaOH
Being a population-based study, motif repeat expansion or Variable Number of Tandem Repeats (VNTR) study was carried on the North Indian population.

1. Subjects
High intrabreeding status due to demographic, cultural and religious preferential options provided a chance of pure gene pool and possibility of preservation of population-based significant genes in North-Indian population. There has been shown strikingly distinct increase in the neuropsychiatric diseases during past decade or so, analysis of patients registered at psychiatric hospitals' show that with decrease in the age onset, there is predisposition of the neuropsychiatric diseases such as Schizophrenia thus making this study of great importance. The information collected for the identification of the patient was proformed as:

1. Patient’s Name.
2. Clinical Diagnosis.
3. Age.
4. Age of onset.
5. Mother’s Name and Age.
7. Number of Litter size.
8. Number in the sib-size.
9. Family member affected, if any.
10. Any other information.
11. Pedigree analysis

1.1 Sampling
To ensure the pure-line sample collection, blood samples of only those normal individuals and Schizophrenic subjects were collected who were of pure North-Indian origin, atleast upto four generations. Both normal and patient age group averaged between 21-50 years.
1.2 Ethnic Status

For broad-spectrum observation, subjects - Normal individuals and Schizophrenic patients representing the greater part of North India were included randomly. Without any ethnic bias, blood samples of subjects from UP, Kashmir, Punjab, were collected. Samples included one male patient from Ladakh and one female patient from a sub-ethnic Gujjar population of Kashmir. Samples were collected strictly in accordance with the institutes' ethical committee, with the informed consent of the donors/volunteers or their guardians in chronic cases for taking blood sample.

2. Collection of Blood

Our study was based on the DNA analysis isolated from blood collected from the forty (40) Schizophrenic patients of pure North-Indian origin - twenty (20) of the subjects were males and twenty (20) females. For comparative study, blood samples from normal twenty (20) male and twenty (20) female subjects also of pure North-Indian origin were collected randomly. The normal individuals included in the study were healthy laboratory volunteers with no history of any psychiatric illness upto four generations. The samples were collected strictly in accordance to the clinical diagnostic for disease criteria (DSM-IV) of the disease established by the consultants and medicos in service. Patient blood samples were made available from Department of Mental Health Care and Psychiatry Hospital, Srinagar, Kashmir, Psychiatric Department of Jawaharlal Nehru Medical College (JNLMC), Aligarh Muslim University, Aligarh.

For DNA isolation, using 0.5M EDTA, approximately 5 ml of peripheral blood sample was collected from each of the subject involved in our study. Blood samples from forty (40) patients – twenty (20) males and twenty (20) females with Schizophrenia anomalies were obtained for studying the polymorphic status of trinucleotide repeat expansions in the Schizophrenic patients specifically. Of these, twenty (20) subjects were suffering from Chronic Schizophrenia and twenty (20) subjects were in the range of Mild-Average type of Schizophrenia, SCZ (Mild-Avg.). Also forty (40) blood samples from healthy individuals with no mental illness records in either themselves or a first-degree relative were collected. Of those samples, twenty (20) were normal males and twenty (20) normal females.
3. Isolation of Genomic DNA

Total genomic DNA was isolated from blood following standard protocol (Miller SA, et al; 1988) as per the high-salt, phenol-chloroform method. Blood was collected in vacutainers containing 100 µl of 0.5M EDTA. Chilled lysis buffer (150 mM NH4Cl, 10 mM KCl, 0.1 mM EDTA) pH 7.8, was added in the ratio of 1:10 and the tubes were kept on ice for a minimum of 15 min. with intermittent gentle mixing. The cell pellet was harvested by spinning the sample at 5000 rpm at 4°C for 15 min. The pellet was resuspended in 5 ml of nuclease buffer (75 mM NaCl and 25 mM EDTA), 0.2%SDS and 0.02mg/ml of proteinase-K. The tubes were incubated at 37°C for 16 hrs to facilitate the digestion of cellular/nuclear proteins by proteinase-K. After digestion, the mixture was subjected to one extraction with equal volume Phenol, followed by two extractions with equal volume of phenol: chloroform (1:1) and finally one extraction with chloroform: isooamyl alcohol (24:1). The DNA (aqueous phase) was precipitated with 0.1 volumes of 3 M sodium acetate (pH 5.2) and 2 volumes of chilled ethanol at room temperature (RT), spooled out into an eppendorf tube and washed twice with 70% ethanol. The DNA samples were dried and finally dissolved in 1:0.1 TE (10 mM Tris HCl; 1 mM EDTA pH 8.0).

3.1 Spectrophotometric Quantitation of DNA

DNA concentration was estimated using spectrophotometric absorbance at 260 nm. DNA samples were reconstituted in 0.1 N NaOH. The concentration of DNA was estimated by using the following formula

\[ \text{concentration} = \text{OD}_{260\text{nm}} \times \text{dilution factor} \times 0.04 \text{mg/ml} \]

where 1 OD = 0.04 mg/ml (for ss DNA and RNA).

* = Multiplication.

3.2 Dilutions of Genomic DNA for PCR Reaction

Dilutions of same concentration (100ng/µl) were prepared from the stock DNA of the patient and normal samples using formula-

\[ N_1 V_1 = N_2 V_2 \]

3.3 Oligonucleotides for Southern Hybridization and PCR

Oligonucleotides were purchased from Biobasic Inc (Canada) and Microsynth (Balgach) and purified on polyacrylamide gel electrophoresis.
The details of all the oligonucleotides used for PCR have been given in Table-9.

4. Polymerase Chain Reaction

4.1 Conditions for PCR Amplification

Reactions were carried out in a total volume of 25 μl as per the following protocol.

- To 25 ng (1 μl) DNA the following was added
  - 10X PCR buffer 2.5 μl
    - (100 mM Tris HCl pH 8.8; 500 mM KCl; 0.8% Nonidet P40)
  - Magnesium chloride 25 mM 1.5 μl
  - dNTP mix 1.25 mM 4.0 μl
  - sense primer ~ 10 picomoles
  - antisense primer ~10 picomoles
  - Taq DNA polymerase 0.25 units

Volume was made upto 25 μl with triple distilled water.

- Gently mix and microfuge to contents for volume accumulation at the base of the tube.
- The mixture was incubated for 95-96°C for 2-15 minutes (Table-10) to denature.
- The subsequent 32-35 cycles included denaturation at 94-96°C for 30 seconds-2 minutes, annealing at appropriate Tm (Table-10) for 30 seconds-1 minute and extension at 72°C (30 seconds-1 minute per kb to be amplified).
- Final extension was carried out at 72°C for 7-10 minutes as per the primer involved.
- The reaction mixture was stored at 4°C until agarose gel electrophoresis.

4.2 Agarose Gel Electrophoresis of Resultant Amplicons

PCR generated amplicons were resolved on 1-2% agarose (depending on the amplicon size) in 1 X TAE buffer containing Ethidium bromide at 20 volts/cm following standard protocols (Sambrook et al., 1989). The amplicon size was determined using a molecular size marker run alongwith the samples. The gel was
Table 9: Primers used to study Polymorphic Repeat Expansions in Schizophrenic patients.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Chromosome</th>
<th>Annealing-Temperature Ta (°C)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sam 1</td>
<td>5'CAG AAA AGC ACC TGG TAC TAA 3' 5'GGG CTG GAG CCT TTT TAC TCG C 3'</td>
<td>12q14.2, 3p22-p21.2</td>
<td>59</td>
<td>116</td>
</tr>
<tr>
<td></td>
<td>5'CAC CAG TCT CAA CAC ATC ACC ATC3' 5'CCT CCA GTG GGT GGG GAA ATG CTC 3'</td>
<td>12p13.31</td>
<td>62</td>
<td>144</td>
</tr>
<tr>
<td>Sam 3</td>
<td>5'CCC TGC CTG AAG AGG GAC AG 3' 5'TCT GGG TCT GAC CAC TGA GAC 3'</td>
<td>6p21.32</td>
<td>62</td>
<td>145</td>
</tr>
<tr>
<td></td>
<td>5'GTC CTC AGT AAC CAA TG 3' 5'AGA GCT GCC AGA AGG TGA 3'</td>
<td>8p22.3-24.1</td>
<td>55</td>
<td>222</td>
</tr>
<tr>
<td>Sam 5</td>
<td>5'CAC CTC TCT TCT TCT AAC CCC 3' 5'CTC GGC CAG TTT TTA CAA CTT G 3'</td>
<td>6q16.3-q21</td>
<td>55</td>
<td>149</td>
</tr>
<tr>
<td></td>
<td>5'CCT GAT GTG CTG ACA CCT GC 3' 5'AAG CTC CCG AAT GTT GTC C 3'</td>
<td>1</td>
<td>55</td>
<td>126</td>
</tr>
</tbody>
</table>

Note:
Oligonucleotides were purchased from Biobasic Inc (Canada) and Microsynth (Balgach).
photographed and the precise molecular weight of the amplicons analysed using gel-documentation system UVIDoc Mw Version 99.03 for Windows.

5. Sequencing Reaction

Sequencing reactions were performed with dsDNA isolated from extraction and subsequent purification of PCR product using Sequenase Version 2.0 PCR Product Sequencing kit (USB) following Sanger's dideoxychain termination method (Sanger et al., 1977).

Template for sequencing was prepared by denaturing 4-5 μg of ds DNA with 0.2 N NaOH and 2 mM EDTA at 37°C for 30 min. DNA was precipitated with 0.1 volume of 3M sodium acetate (pH 5.2) and 2.5 volumes of pre-chilled ethanol at -70°C for 15 min. The tube was centrifuged at 13,000 rpm for 15 min. followed by two washes in 70% ethanol. The pellet was dried and dissolved in 7 μl of triple distilled water and left at RT for 30 min. Sequencing reaction was conducted as per the following protocols.

- To 5 μl of PCR amplified dsDNA was added 1μl of exonuclease I (10U/ml in 20mM Tris-HCl, pH 7.5, 5mM 2-mercaptoethanol, 50% glycerol) and 1μl shrimp alkaline phosphatase (2U/ml in 25mM Tris-HCl, pH 7.6, 1mM MgCl2, 0.1mM ZnCl2, 50% Glycerol). Mix and incubate the mixture at 37°C for 15 min. This cleansed the PCR product before sequencing.
- exonuclease I and shrimp alkaline phosphatase were inactivated by heating to 80°C for 15 mins.
- Annealing was carried out in annealing buffer (40 mM Tris-Cl, pH 7.5; 20 mM Magnesium chloride; 50 mM NaCl) with 5 μl ds DNA, 1μl primer in a total reaction volume of 10 μl at 100°C for 2-3 min.
- The mixture was cooled quickly by placing the vial directly in an ice-bath for 5 min. Sequenase version 2.0 DNA polymerase enzyme (1 μl) was diluted 8 fold in enzyme dilution buffer (10 mM Tris-Cl, pH 7.5; 5 mM DTT; 0.5 mM(EDTA),50%glycerol).
- The dGTP labelling mix (1.5 μM dGTP, dCTP, dTTP) was diluted 5 times in triple distilled water.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Pre-PCR cycles, Denaturation °C mins./sec</th>
<th>PCR cycles °C mins/secs</th>
<th>Post-PCR cycles, Extension °C mins./secs</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sam 1</td>
<td>96°C -2mins</td>
<td>denaturation 96°C -1min annealing 59°C -1min extension 72°C -1min</td>
<td>72°C -10mins</td>
<td>32</td>
</tr>
<tr>
<td>Sam 2</td>
<td>96°C -2mins</td>
<td>denaturation 96°C -2min annealing 62°C -1min extension 72°C -1min</td>
<td>72°C -10mins</td>
<td>32</td>
</tr>
<tr>
<td>Sam 3</td>
<td>95°C -15mins</td>
<td>denaturation 94°C -30 secs annealing 62°C -30 secs extension 72°C -30 secs</td>
<td>72°C -10mins</td>
<td>35</td>
</tr>
<tr>
<td>Sam 4</td>
<td>96°C -2mins</td>
<td>denaturation 96°C -2 min annealing 55°C -1 min extension 72°C -1 min</td>
<td>72°C -10mins</td>
<td>32</td>
</tr>
<tr>
<td>Sam 5</td>
<td>96°C -2mins</td>
<td>denaturation 96°C -2 min annealing 55°C -1 min extension 72°C -1 min</td>
<td>72°C -10mins</td>
<td>32</td>
</tr>
<tr>
<td>Sam 6</td>
<td>95°C -5mins</td>
<td>denaturation 96°C -2 min annealing 55°C -1 min extension 72°C -1 min</td>
<td>72°C -7mins</td>
<td>35</td>
</tr>
</tbody>
</table>
To the 10 μl of ice cold annealed DNA mixture 1 μl DTT (0.1 M), 2 μl of diluted dGTP mix, 2 μl of diluted sequenase enzyme (3 units) and 5 μCi [α-35S] dATP typically 0.5 μl of 3000Ci/mM was added and incubated at RT for 5 min.

Simultaneously 2.5 μl of each ddNTP termination mix (8 μM of one ddNTP in addition to 80 μM dATP, dTTP, dCTP and 50 mM NaCl) was dispensed into four separate eppendorfs labelled as G, A, T, C and prewarmed at 37°C for 5 min.

3.5 μl of labelling reaction was added to each of the four termination tubes mixed and incubated at 37°C for 5 min.

Reactions were terminated by adding 4 μl of stop solution (95% Formamide; 20 mM EDTA; 0.05% Bromophenol blue; 0.05% Xylene Cyanol FF), mixed thoroughly and stored at -20°C until loading on the sequencing gel.

6. Polyacrylamide Gel Electrophoresis

For resolving sequencing reaction, 6% polyacrylamide gel was prepared as follows

- Acrylamide 5.7 g
- Bis acrylamide 0.3 g
- Urea 48 g
- 10X TBE 10 ml
  - (890 mM Tris-Cl; 890 mM Boric acid; 2 mM NaEDTA, pH 8.2)
- Distilled water 40 ml

The acrylamide solution was prepared fresh and volume adjusted with water to 100 ml. Sequencing gel apparatus, (Bio-Rad, USA) of 38 cm X 50 cm with 0.4 mm spacers and 20 well 0.4 mm comb was used. The gel was prepared using 40 ml of acrylamide solution with 225 μl of 10% APS and 25 μl of TEMED and allowed to polymerize for 2-3 hours.

The gel was pre-electrophoresed for 1 hour at 1900 constant volts to achieve the 50°C temperature. Prior to loading the reaction mixture on gel, samples were heated at 90°C for 10 min. and 5 μl of each sample was loaded for electrophoretic separation at the above mentioned temperature. After electrophoresis, the gel was
transferred onto 3MM Whatman sheet, covered with saran wrap and dried at 80°C under vacuum (Savant, Gel drying system). Gel was exposed to X-ray films (Konica, India or Hyperfilm β-max, Amersham) at RT for 16-48 hours. Developing and fixing of autoradiograms was done following the protocols described earlier.

PCR products were used for automated sequencing on the commercially available automated sequencer (ABI, 377) using automated sequencing facility at Eukaryotic Gene Expression Laboratory, NII.

6.1 Sequence Analysis
The entire per contig of Sam1, Sam2, Sam3, Sam4, Sam5, and Sam6 were analysed for the presence of short tandem repeats using programs as DNAsis (LKB/Pharmacia, Sweden) and GeneRunner programme (Hastings Software, USA). A database search was performed on the NIH mail server using BLASTX, BLASTN and FASTA programs for the sequence homology with the others in the Genbank using sites on default blast server as http://www.ncbi.nigh.gov.cgi-bin/Blast/nph-newblast.

Some of the details pertaining to the sequence similarity as per our search were—

**Sam1/CTGB33**
Also known as: L14; RL14; hRL14; CTG-B33; MGC88594; CAG-ISL-7
UniGene Hs.: 446522
GDB: 373727.
UnistS: 86111
Gene ID: 9045 (updated 21-Nov-2007)
Genbank Accession No: D87735
Definition: 60S ribosomal protein L14 (CAG-ISL-7).
Databases: Uniprot/SWISSPROT P50914
Official Symbol: RPL14
Official Full Name: ribosomal protein L14
Primary source: HGNC: 10305

**Sam 2 /CTGB37**
Also known as: DRPLA, ATN1
UniGene Hs: 143766 Atrophin 1
GDB: 293944
UniSTS: 148888
Gene ID: 1822
Genbank Accession No: D12S755E
Definition: Found by e-PCR in sequences from Homo sapiens and Pan troglodytes at chromosome 12, locus ATN1
Databases: EMBL, SWISSPROT, TREMBL, WORMPEP.
Official Symbol: ATN1
Official Full Name: Atrophin 1
Primary source: Atrophin 1

Sam 3 /NOTCH4
Also known as: DAQB-143M3.4, FLJ16302, INT3, MGC74442, NOTCH3
UniGene Hs: 436100
GDB: G00-364-353
UniSTS: 54644
Gene ID: 4855
Genbank Accession No: NM_004557
Definition: Homo sapiens Notch homolog 4 (Drosophila) (NOTCH4).
Databases: HCOP H-InvDB, Treefam Vega, Q99466, SwissProt, UniProt
Official Symbol: NOTCH4
Official Full Name: Notch homolog 4 (Drosophila) [Homo sapiens]
Primary source: HGNC: 7884.

Sam4/AAT21 (DTNBP1)
Also known as: SDY, DBND, HPS7, My031, FLJ30031; MGC20210, DKFZP564K192
UniGene Hs: 571148
GDB: 11505970
UniSTS: -
Genbank Accession No: NM_183040
Version: NM_183040.1 GI: 34304367
**Definition:** Human DNA sequence from clone RP3-322I12 on chromosome 6p22.3-24.1

**Databases:** EMBL, SWISSPROT, TREMBL, WORMPEP, UniProt.

**Official Symbol:** DTNBP1 provided by HGNC

**Official Full Name:** dystrobrevin binding protein 1 (provided by HGNC)

**Primary source:** HGNC: 17328

**Sam5/GLUR6**

**Also known as:** EAA4, GRIK2 EAA4, GLR6, MRT6, GLUR6, MGC74427

**UniGene Hs:** 98262

**UniSTS:** 156925

**Gene ID:** 2898

**GDB:** 373727

**Genbank Accession No:** NM_175768

**Version:** NM_175768.1 GI: 28559002

**Definition:** triple transcript variants encoding distinct isoforms identified for this gene

**Databases:** Uniprot/SWISSPROT

**Official Symbol:** GRIK2

**Official Full Name:** Glutamate receptor, ionotropic, kainate 2

**Primary source:** HGNC: 4580

**Sam6/CTGB1**

**Also known as:** FLJ37346, KIAA1150, MGC138257, MGC138285, P66beta, RP11-216N14.6

**UniGene Hs:** 4779

**UniSTS:** 463760

**Gene ID:** 57459 (updated 13-Nov-2007).

**GDB:** AF411836

**Genbank Accession No:** NM_020699

**Definition:** transcription repressor p66 beta component of the MeCP1 complex

**Databases:** SwissProt, UniProt

**Official Symbol:** GATAD2B

**Official Full Name:** GATA zinc finger domain containing 2B [Homo sapiens]
7. Statistical Analysis

The frequency distributions of the genotypes and alleles studied between patients and normal controls were statistically compared using Chi square test and Fishers exact test. Analysis was done using SPSS version 10.01 for Windows. The significance of the differences in Alleles and Genotype frequencies found on comparative basis in the Intra-gender studied populations'- Normal Male and Schizophrenic Male, Normal Female and Schizophrenic Female and Inter-gender population- Schizophrenic Male and Schizophrenic Female was calculated in aggregate by Chi square test and at individual frequencies with Fishers exact test. Same analytic tests were applied to study significance of TNR polymorphism between Schizophrenic (Mild-Avg.) and Chronic Schizophrenia. Our study on North-Indian population agreed to the polymorphic status of all the loci as had been reported by many earlier studies on different populations (Caucasian, Japanese, Chinese). Distribution of VNTR (Variable Number of Tandem Repeats) alleles or microsatellites in all the comparative population studies we had undertaken are shown in tables I- 1-5 and II- 1-5.