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Mutation screening of two candidate genes from 13q32 in families affected with Bipolar disorder: human peptide transporter (SLC15A1) and human glypican5 (GPC5)

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Abstract

Background: Multiple candidate regions as sites for Schizophrenia and Bipolar susceptibility genes have been reported, suggesting heterogeneity of susceptibility genes or oligogenic inheritance. Linkage analysis has suggested chromosome 13q32 as one of the regions with evidence of linkage to Schizophrenia and, separately, to Bipolar disorder (BP). SLC15A1 and GPC5 are two of the candidate genes within an approximately 10-cM region of linkage on chromosome 13q32. In order to identify a possible role for these candidates as susceptibility genes, we performed mutation screening on the coding regions of these two genes in 7 families (n=20) affected with Bipolar disorder showing linkage to 13q32.

Results: Genomic organization revealed 23 exons in SLC15A1 and 8 exons in GPC5 gene respectively. Sequencing of the exons did not reveal mutations in the GPC5 gene in the 7 families affected with BP. Two polymorphic variants were discovered in the SLC15A1 gene. One was T to C substitution in the third position of codon encoding alanine at 1403 position of mRNA in exon 17, and the other was A to G substitution in the untranslated region at position 2242 of mRNA in exon 23.

Conclusions: Mutation analysis of 2 candidate genes for Bipolar disorder on chromosome 13q32 did not identify any potentially causative mutations within the coding regions or splice junctions of the SLC15A1 or GPC5 genes in 7 families showing linkage to 13q32. Further studies of the regulatory regions are needed to completely exclude these genes as causative for Bipolar disorder.

Background

Schizophrenia and associated mental illness are complex psychiatric disorders each affecting about 1% of the world

population. Genetic evaluation of Schizophrenia and associated mental illness have suggested an important role for genetic factors [1-4], however, no specific gene or bi-

ological marker has yet been identified. Genome scans have mapped several loci in various ethnic groups. Replications of these scans were positive in some cases and negative in others [2,5,6]. These data as well as reports of the specific candidate genes beginning to be identified implicate genetic heterogeneity of susceptibility genes [7,8] and also sex specific differences [9] in the molecular basis of disease phenotype. Linkage analyses of all published genome scans, for Schizophrenia genes, have identified chromosome 13q32 as one of the regions with evidence of linkage to Schizophrenia. In independent genome scans, Bipolar disorder has also shown linkage to this region [10–13]. The linkage region for both disorders spans approximately 10 cM on chromosome 13q32–q33 [14]. The region between D13S71 and D13S274 showed linkage to Bipolar disorder based on a whole genome scan with an average marker spacing of ~6 cM on 13q32. Fine mapping of this region, using nine additional markers, with an average spacing of 0.9 cM confirms the evidence of linkage to 13q32 around markers D13S79–D13S225 [15]. Association analyses with GASSOC TDT and ASPEX/Sib_tdt showed linkage disequilibrium signals with several markers, including D13S280 which need follow up by typing more markers in the region in a larger sample size [15]. Meta analysis of whole-genome linkage scans further strengthens the susceptibility locus of BP on chromosome 13q32 [13]. All these studies clearly point towards further efforts in the identification of susceptibility genes for this disorder. Based on evidence of linkage, a comprehensive linkage map was developed around this region [14]. Complete annotation of this region may include as many as 200 genes. One approach to identify susceptibility locus is to use the candidate gene approach. Studies are underway at our center to identify susceptibility variants by mutation screening of candidate genes in this region on a small series of Bipolar families showing linkage to 13q32. For the present study we have selected two candidate genes viz. human peptide transporter (SLC15A1) and human glypican5 (GPC5) for mutation screening from this region.

SLC15A1 is an intestinal oligopeptide transporter also referred to as HPEPT1 that belongs to solute carrier family15. It is expressed in intestine, kidney, liver and brain [16]. The function of SLC15A1 is to absorb and transport small peptides [17,18]. The efficiency of absorption and transportation of L DOPA and its metabolite dopamine, which are neurotransmitter candidates, is increased by SLC15A1 [19]. Therefore, it is an important potential gene for future drug design, delivery, and targeting [20].

GPC5 belongs to the cell surface heparan sulfate proteoglycan family and is expressed in limb, kidney, developing central nervous system and brain tissue [21,22]. In

vertebrates 6 glypican family members belonging to the proteoglycan family have so far been characterized [23]. Although there is a high degree of conservation of polypeptide sequence among family members, they are localized to different chromosomes. Glypican5 is structurally correlated to glypican3 and localized to chromosome 13q32. Deletion of the 13q region is associated with human 13q syndrome, a developmental disorder [24]. Some of the proteoglycans play important functional roles in neurogenesis, axon guidance, synapse development [25], and cellular growth and differentiation [26,27,23]. Mutations in GPC3 cause Simpson-Golabi-Behmel syndrome [28,29], however, no mutations were detected in the GPC5 and GPC6 genes [30]. The glypican1 gene has been suggested to be a good candidate for brachydactyly type E [31]. Given that, GPC5 is highly expressed in brain and mutations in other genes of the glypican families are associated with developmental disorder; GPC5 could be a potential candidate gene for Bipolar disorder.

In order to identify a possible role for these candidates (SLC15A1 & GPC5) as susceptibility genes, we performed mutation screening of the coding regions in 7 families (n = 20) affected with Bipolar disorder showing linkage to 13q32. First, the accurate gene structure of SLC15A1 & GPC5 was defined by analysis of the available draft human genomic sequence. Next, PCR primers and conditions for amplification and sequencing of each exon were developed and sequencing was performed.

Results

Genomic structure

We determined genomic structure of both the genes by aligning cDNA sequences with available human draft genomic sequences <http://www.ncbi.nlm.nih.gov/>. The entire SLC15A1 gene was represented in one BAC clone sequence AL357553 and GPC5 in 6 BAC clone sequences. Alignment of SLC15A1 cDNA sequence (accession number U21936) with the genomic sequence (accession no. AL357553) revealed that it is a large gene with a coding sequence of 3105 bp long, having 23 exons spread over ~68.85 kb of genomic sequence. The splice junctions follow the GT/AG rule (Table 1).

Alignment of GPC5 cDNA (accession number U66033) with the working draft sequence revealed a genomic organization of 8 exons, which was represented in 6 BAC clone sequences. The coding sequence of GPC5 gene is 2558 bp long and encodes 572 amino acids. The first two exons of the GPC5 gene are present in BAC clone accession number AL138714, exons 3,4,5 in AC027429, exon 6 in AL162456, exon 7 in AL163537 and exon 8 in AL157363 (Table 2). Most of the splice junctions follow the GT/AG rule.

Table 1: Genomic structure of human peptide transporter (SLC15A1) showing number of exons, cDNA position, exon size, exon-intron junctions, 3' & 5' intron acceptor & donor sites, intron size and intron phase.

| Exon | cDNA bp Position | Exon Size | Intron-Exon Junctions | | | Intron Size bp | Intron Phase |
|------|------------------|-----------|-----------------------|---------------------|-----------|----------------|--------------|
| | | | Intron 3' | Exon | Intron 5' | | |
| 1 | 2-61 | 61 | CACCTG...CCATGG | gtacgctc | >25 kb | 1 | |
| 2 | 61-77 | 16 | tccttttag | GAATGTTACAC | 394 | 2 | |
| 3 | 78-159 | 81 | gtcga | AGTTTTCTTT...TGCGAG | 102 | 1 | |
| 4 | 158-302 | 144 | tgcaaaag | CAATCC...GTTCAA | 2091 | 2 | |
| 5 | 302-422 | 120 | aaacacag | GACCAT...GACAGT | 2008 | 2 | |
| 6 | 422-522 | 100 | ttcccag | GTTGCT...GGCCAG | 215 | 0 | |
| 7 | 519-611 | 92 | cattgcag | GAGAAA...TCAGAG | 2173 | 2 | |
| 8 | 611-696 | 85 | ctccccag | TTC AAC...CCCTGAG | 3277 | 1 | |
| 9 | 697-779 | 82 | ctatctag | TTGTGT...ATCGGT | 3294 | 0 | |
| 10 | 780-867 | 87 | ttccttcag | TTTGCC...TACGAT | 554 | 0 | |
| 11 | 867-957 | 90 | aactacag | GAGCGG...CAGCAG | 1940 | 0 | |
| 12 | 955-1002 | 47 | atttttaag | GGCTCC...GAAATC | 92 | 0 | |
| 13 | 1002-1034 | 32 | tccttttag | GGAGCTATGCAG | 77 | 0 | |
| 14 | 1030-1123 | 93 | cctctcag | ACCGTG...TTTCAC | 801 | 2 | |
| 15 | 1120-1205 | 85 | tgccatcag | CTCCTT...ATCGAT | 2433 | 0 | |
| 16 | 1206-1325 | 119 | ttgtctcag | AAAAC...TCTCAA | 1699 | 0 | |
| 17 | 1326-1476 | 150 | ctctatag | ACAAAT...CAGGTG | 1755 | 2 | |
| 18 | 1472-1522 | 50 | tctctcag | GTAAG...AATCAG | >13 kb | 2 | |
| 19 | 1519-1630 | 111 | tgcttcag | ATTTGT...TGGCAT | 114 | 2 | |
| 20 | 1631-1739 | 108 | ttgatag | AAAAG...AGGAAG | 522 | 0 | |
| 21 | 1738-1884 | 146 | tgctgcag | AATGAC...TCTCAG | 1279 | 0 | |
| 22 | 1879-1991 | 112 | ttcctcag | GCTCCT...AAACAG | 1273 | 0 | |
| 23 | 1990-3105 | 1115 | cttcaaag | TGGGCC...CTGTTCTTA | | | |

Mutation analysis

No mutations were identified in the GPC5 gene in the 7 families affected with Bipolar disorder. Two polymorphic variants were discovered in SLC15A1 gene, one in exon 17 and another one in exon 23. Sequence analysis revealed a T to C (Figure 1A) substitution in third position of codon encoding alanine at 1403 position of mRNA and A to G (Figure 1B) substitution at position 2242 of mRNA. Of these, 6 individuals (3 affected and 3 normal) were homozygous CC (exon 17), 15 (11 affected and 4 normal) were heterozygous TC (exon 17) and three were heterozygous AG (exon 23).

Discussion

Gene mapping studies using microsatellite markers have identified chromosome 13q32 as one of the putative loci for Schizophrenia and Bipolar disorder [10-13,15]. To begin identification of a susceptibility gene for Bipolar disorder, we have screened two candidate genes, SLC15A1 and GPC5, on the basis of their genetic localization within the 10 cM region of chromosome 13q32 [14]. The entire SLC15A1 gene was represented in one BAC clone sequence AL357553 and GPC5 in 6 BAC clone sequences. Alignment of SLC15A1 cDNA sequence with genomic sequence (accession no. AL357553) revealed that it is a large gene with a coding sequence of 3105 bp long, having 23

exons spread over ~68.85 kb of genomic sequence. The splice junctions follow the GT/AG rule (Table 1). A wide variety of transporters are found in the intestine and several genetic disorders have been shown to result from deficient intestinal transporters [20]. SLC15A1 consists of 708 amino acids with putative 12 transmembrane domains and 2 putative protein kinase C phosphorylation sites. Exon-intron boundaries occur mostly in the loops connecting transmembrane segments [32] suggesting a modular gene structure reflecting the TMS-loop repeat units in SLC15A1. Computer modeling and site directed mutagenesis studies suggested a list of amino acids for the transport functional activities. Tyrosine (Y167) and histidyl residues (H57, H111 and H121) are two of these amino acids playing important roles in the function of transporters [33-36]. In our mutation screening of families with Bipolar disorder we did not find variants at these positions. However, we did identify two variants in the SLC15A1 gene, one in exon 17 and another one in exon 23. Sequence analysis revealed a T to C substitution in the third position of codon encoding alanine at 1403 position (exon 17) of mRNA. Of these, 6 individuals were homozygous CC (29%) and 15 were heterozygous TC (71%) (exon 17). However, there was no amino acid change. Substitution of T to C in normal members as well, suggests it is a polymorphic variant and not a pathogenic mu-

Table 2: Genomic structure of human glypican5 (GPC5) showing number of exons, cDNA position, BAC clones representing genomic sequence, exon-intron junctions, 3' & 5' intron acceptor & donor sites, intron phase and exon size.

| Exon | cDNA bp position | BAC clone | Intron-Exon Junctions | | | Intron phase | Exon size |
|------|------------------|-----------|----------------------------------|-------------------------|-----------------|--------------|-----------|
| | | | Intron 3' acceptor | Exon | Intron 5' donor | | |
| 1 | 1-178 | AL138714 | | GACGGC...GGGCAGgtaagggg | | 1 | 177 |
| 2 | 175-339 | AL138714 | gtgtccagGACCTG..TTCAAGgtgatct | | | 1 | 164 |
| 3 | 338-1044 | AC027429 | tgttacagAAACCC...GAACAGgtaagtag | | | 0 | 706 |
| 4 | 1033-1168 | AC027429 | ttaaaagGTAAAT...AAGAAAgtaagaca | | | 1 | 135 |
| 5 | 1169-1294 | AC027429 | tcatttagAGAATT...AAAAAGgtatttta | | | 2 | 129 |
| 6 | 1293-1415 | AL162456 | cttgctagTTATAC...GTTTCAGgtaagtcc | | | 0 | 122 |
| 7 | 1414-1576 | AL163537 | acaattagTTGTTA...CAGACTgtaagtgt | | | 1 | 162 |
| 8 | 1576-2526 | AL157363 | ctctacagGGATGCC...TGGTGG | | | | 950 |

tation. In exon 23, the variation observed was an A to G substitution in the untranslated region at position 2242 of mRNA. Of these, eighteen were homozygous AA (86%) and three were heterozygous AG (14%). The variant in exon 17 was also found in the SNP database (NCBI SNP cluster ID rs1339067) although the population frequency was not reported. An association study of this variant in a large population will aid in understanding the potential role of the SLC15A1 gene in Bipolar disorder.

Alignment of the GPC5 cDNA with the working draft sequence revealed a genomic organization of 8 exons, which was represented in 6 BAC clone sequences (Table 2). The whole GPC5 gene is 2558 bp long and encodes 572 amino acids. The first two exons of the GPC5 gene are present in BAC clone accession number AL138714, exons 3,4,5 in AC027429, exon 6 in AL162456, exon 7 in AL163537 and exon 8 in AL157363 (Table 2). Most of the splice junctions follow the GT/AG rule. The exon structure was confirmed by comparing the cDNA with nucleotide contig NT_009866. While we finished screening this gene for mutations, its genomic structure was published and confirms the presence of 8 exons with exon/intron boundaries following the AG/GT rule with a size of approximately 2 Mb [30]. No mutations or polymorphisms were identified in the GPC5 gene in the 7 families affected with Bipolar disorder decreasing the likelihood that this gene is involved in the etiology of Bipolar disorder. Several disorders have been reported due to mutations in glypican gene families [31,37] and interestingly due to deletion of region on chr.13 q32 [24,38]. It would be interesting to study the occurrence of Schizophrenia or other associated mental illnesses in individuals with these deletions. Mutation screening of the glypican genes in these patients might give clue to Schizophrenia susceptibility locus.

Absence of pathogenic mutations in the two genes GPC5 and SLC15A1 studied in seven families of Bipolar disorder

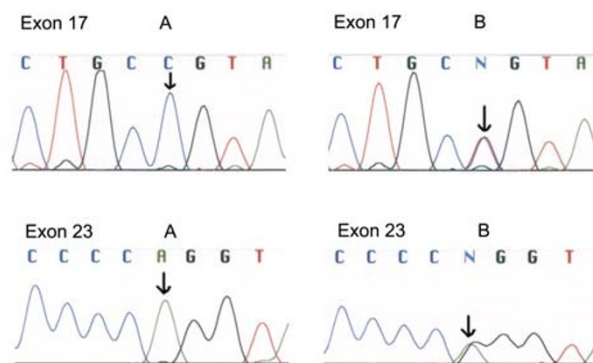


Figure 1
Sequencing traces demonstrating polymorphic variants in exon 17(T→C) and exon 23 (A→G) of SLC15A1 gene. Exon 17 A: Homozygous CC, Exon 17 B: Heterozygous TC, Exon 23 A: Homozygous AA, Exon 23 B: Heterozygous AG.

suggests that these genes are presumably not involved in the susceptibility locus for Bipolar disorder in the families screened. However, due to the complex nature of these diseases, no definite conclusions can be drawn. We cannot rule out genetic heterogeneity for these genes and suggest studying more families. For example, the NOTCH 4 gene that is located on chromosome 6p21.3 confers susceptibility to Schizophrenia in the British population but not in the Japanese population [39]. Genetic heterogeneity is very common in hereditary deafness [40]; also the prevalence of mutations in the connexin 26 gene is a major cause of non-syndromic autosomal recessive deafness that varies in different populations [41].

Table 3: Human peptide transporter (SLC15A1) : PCR Primers and Annealing temperature

| Exon | cDNA bp | Position | Forward Primer Primer sequence 5' → 3' | Reverse Primer Primer sequence 5' → 3' | Product Size bp | Annealing Temp. |
|------|-----------|----------|---|---|-----------------|--------------------|
| 2 | 61–77 | P17 | ccctctgaccaccctaaaaa | tgaacccttaggggtaaaaca | 191 | 60 |
| 3 | 78–159 | P1 | tggggaaggattagttaggg | aactttccagccacgagt | 498 | 60 |
| 4 | 158–302 | | | | | |
| 5 | 302–422 | P2 | tgtggtggagtcaaaagtgg | cctgaagaccagctcaatc | 356 | 60 |
| 6 | 422–522 | P3 | gtcactatgccaggccact | gcctctgactcctggatgtg | 258 | 60 |
| 7 | 519–611 | P18 | agtggattgatagccaaagtcatct | aagacacggacttggcctta | 200 | 60 |
| 8 | 611–696 | P4 | tgtgaaagcaatcgttaattatcag | ctacttttggccactgttacat | 295 | 60 |
| 9 | 697–779 | P16 | tcaagagccatttctattcttcc | gcatctcttaggccacagg | 350 | 48 |
| 10 | 780–867 | P5 | tgaaatgtgcttccctgaca | tactgacattttgtccatgt | 254 | 60 |
| 11 | 867–957 | P6 | acctccagcttgcctcta | tgaagtggccttggactctg | 293 | 60 |
| 12 | 955–1002 | P7 | cagggtactgctttgtgacg | tatcctctaggcagggttc | 701 | 60 |
| 13 | 1002–1034 | | | | | |
| 14 | 1030–1123 | | | | | |
| 15 | 1124–1205 | P8 | aagatggggagagtgcttt | gctccaggctcagtttaca | 348 | 60 |
| 16 | 1206–1325 | P9 | aggctctgtgattggcagctt | gctggccttggcatttatac | 379 | 60 |
| 17 | 1326–1476 | P10 | aaacctcatgacgggtctctg | ttttggcctcagatcaca | 400 | 60 |
| 18 | 1472–1522 | P11 | gcctccagagcccttcta | tcagccagccttacattgct | 331 | 60 |
| 19 | 1519–1630 | P12 | gacattgtggcgaatctct | gggagattggccaactca | 356 | 60 |
| 20 | 1631–1739 | | | | | |
| 21 | 1738–1884 | P13 | gtcccatcagcattttctgc | ggccaactcaatttacctgttcg | 292 | 60 |
| 22 | 1879–1991 | P14 | ccatgatgaccatgaacagg | cattgaggccacctgacttt | 299 | 60 |
| 23 | 1993–2315 | P15 | ccaagaacatgtacacca | aggctgaggcaggagaatta | 540 | 60 |

Table 4: Human glypican5 (GPC5) : PCR Primers and Annealing temperature

| Exon | cDNA U66033 | Forward Primer 5' → 3' | Reverse Primer 5' → 3' | Product Size | Annealing Temperature |
|------|-------------|-------------------------|-------------------------|--------------|--------------------------|
| 1 | 1–178 | cgctgtgcttccacgtct | ctaccgcaccaagcatc | 410 | 60 (5%DMSO) |
| 2 | 175–339 | gtgcaggactggcataacg | gtttcccaatccaactcag | 455 | 60 |
| 3 | 338–1044 | agcagatggacgggttttagc | caagaagtcagactgaaaatgtg | 797 | 60 (DT) |
| 4 | 1033–1168 | gcataccacataaatgtccatga | ggcttactttcttcttcttctgg | 408 | 60 |
| 5 | 1169–1294 | ttgatggcctttattgtgga | tcaggttttgttcttcttcc | 352 | 48 |
| 6 | 1293–1415 | gaggaaattccgacctcaaa | cactgcatattgactgcatcc | 401 | 60 |
| 7 | 1414–1576 | ccatttccaattcctctg | tgtaagactttgccgctatt | 466 | 60 |

Considering the complex nature of the disease and the supportive evidence of linkage to chromosome 13q32 [13,15] it would be worth while to continue further studies to find out Schizophrenia susceptibility genes at this critical region. As the human genome reference sequence is nearing completion, the analysis of genetic variation is becoming important. Our plans are to annotate all the genes for a 10-mega base region on chromosome 13 and develop a high density SNP map. Aim is to identify variants in the critical region, to predict haplotypes from numerous variants observed and perform mutation analysis by direct sequencing with the ultimate objective of identifying specific sequence/haplotype associated with the disease phenotype.

Methods

Study Subjects

A total of 7 nuclear families (n=20) affected with Bipolar disorder were selected. The seven families consist of five normal parents, five affected parents, seven affected patients, one normal sib and two affected members. These families were selected from four known pedigree series with evidence of linkage to chromosome 13q32[15].

Genomic structure determination

The genomic structures of SLC15A1 (accession number U21936) and GPC5 (accession number U66033) were determined by direct comparison of their full-length cDNA with the sequence of human chromosome 13 genomic

clones retrieved from the GenBank htgs database <http://www.ncbi.nlm.nih.gov/> by BLASTN searching. Intronic oligonucleotide primer pairs flanking the coding fragments were designed for both the genes using program primer 3 <http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi> using the genomic sequences of SLC15A1 and GPC5 gene respectively.

Mutation analysis

Mutation screening of the coding region of the genes for sequence variants was performed by PCR amplification of 20 samples of 7 BP families and a control sample. Each sample was analyzed for exon 2–23 of the SLC15A1 gene (Table 3) and 7 coding fragments of the GPC5 gene (Table 4). Twenty two fragments of the SLC15A1 gene were amplified in eighteen reactions P1-P18 (Table 3). PCR reactions were carried out under the standard PCR conditions. In brief each 50- μ l reaction contained 50 ng of genomic DNA, 20 pmole of each primer, 1 unit of AmpliTaq Gold DNA polymerase, 5 μ l of 10 X buffer, 2.5 μ l of 2.5 mM of MgCl₂ & 2.5 mM of dNTPs. Amplification conditions were 94°C/ 12 min, 45 cycles of 94°C/30 s, 60°C/ 30 s, 70°C/ 90 s followed by final extension step of 70°C/ 5 min. Sequences of the primers and annealing temperature of each amplification are given in Tables 3 & 4. PCR products were analyzed using 2% agarose gel electrophoresis and purified by 96 well Qia Quick PCR purification kit (Qiagen). Sequencing of each exon except exon 3 of GPC5 was performed using BODIPY dye primer chemistry [42] on an ABI 3700 DNA sequencer (Applied Biosystems, Foster City, CA, USA). Exon 3 of GPC 5 was sequenced with ABI Big Dye terminator chemistry. The obtained sequences were compared with the corresponding control gene sequences using SEQUENCHER software (Gene codes corporation, Ann Arbor, MI, USA).

List of abbreviations

Human peptide transporter: SLC15A1

Human Glypican5: GPC5

Authors' contributions

MM carried out mutation screening of the candidate genes and drafted the manuscript. SLC participated in the development of ~15 Mb map region on human chromosome 13q32. CYL & Author 4, SD Participated in initial genotyping of the families. JAB did the statistical analysis to select the pedigrees. ESG and RAG are PI and CO PI of the project and participated in its design and coordination.

Addendum

While screening an additional three families, and reanalysis of our previous families we noted two variants in exon three: One was A155V (C>T) and second was R223C (C>T). Of the 23 subjects studied, 5 (3 affected and 2 nor-

mal) were heterozygous Ala/Val. 1 (affected) homozygous Val/Val and 17 (13 affected and 4 normal) were homozygous Ala/Ala. The second variant R223C was observed in 2 (1 affected and 1 normal) subjects, both were heterozygous Arg/Cys, 16 affected and 5 normal subjects were homozygous Arg/Arg and none were Cys/Cys homozygous. An association study of the variants detected in GPC5 and SLC15A1 genes in a large gene population will aid in understanding the potential role of these genes in Bipolar disorders.

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