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GENETIC MARKERS OF AUTISM

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ABSTRACT

Autism is a complex neurodevelopmental disorder with a significant genetic component. The prevalence of autism has been increasing globally, though exact statistics for India are not available. Several genetic markers for autism have been studied. These include chromosomal abnormalities, copy number variations, submicroscopic cytogenetic anomalies, single nucleotide polymorphisms and other point mutations. This review gives details on the current data available on these genetic markers of autism, with a focus on single nucleotide polymorphisms. Studies on SNPs within candidate genes on each chromosome are dealt with, including some details on which populations show which variation. Methodology involved in analysis of SNPs, i.e. techniques in SNP genotyping are also reviewed, focusing on those techniques that are simple and economically feasible in the Indian scenario.

KEYWORDS: autism, gene, SNP, genotyping, computational SNP analysis.

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INTRODUCTION

Autism is a complex, behaviorally defined, static disorder of the juvenile brain (age of onset is 18- 24 months [1]) that falls under the category of Autism Spectrum Disorders- a group of neurodevelopmental disorders with- impairments in social interaction, communication, and restricted repetitive patterns of behavior and interests- that include Aspergers's syndrome, disintegrative disorder, Rett syndrome and PDD-NOS (pervasive developmental disorder- not otherwise specified). These are multifactorial (except Rett syndrome, which is a single gene disorder). The prevalence of autism has been rising globally mainly due to better diagnostics. 2 million people are estimated to have autism in India, although detailed statistics are not available. [2, 3, 4, 5] Based on cause, autism can be classified into idiopathic (90% cases, with cause unknown) and secondary (genetic or environmental cause). Genetic causes have been found to play a major role (as demonstrated by concordance studies in twins and siblings), but environmental factors like toxins, teratogens, prenatal infections and injuries are also causative. [6, 7, 8, 9, 10, 11, 12]. This review deals with on the various known genetic markers of autism and the methods available to genotype SNPs.

GENETIC MARKERS

Chromosomal Abnormalities

Standard karyotyping procedures have shown chromosomal abnormalities in some autistic individuals. However, in most of these studies, sample selection did not follow strict guidelines. [7] 3.25 % of autistic individuals have unbalanced translocations, inversions, rings, interstitial deletions, duplications etc. The most prevalent is duplication in chromosome 15q11-13, usually maternally inherited. Other frequent findings are deletions in 2q37, 7q31 and deletions or duplications of 22q13. Klinefelter's Syndrome (XXY), duplications of the Williams–Beuren–Syndrome region 7q11.23 and deletions of 22q11 (Velo-cardio-facial Syndrome) are also associated with increased autistic phenotypic traits. [7]

Copy number variations (CNVs)

Some CNVs are more frequent in autism patients than in controls. Replicated CNVs from genome wide association studies (GWAS) are located on- 1q21, 2p16.3 (NRXN1), 3p25-26 (CNTN4), 7q36.2 (DPP6), 15q11-13 (UBE3A, OR4M2, OR4N4), 16p11.2 (MAPK3, MAZ, DOC2A, SEZ6L2, HIRIP3, IL6) and 22q11.2. [7] A study using SNP arrays in the Netherlands shows that CNV of microcephalin 1 gene (MCPH1) in the 8p23.1 region can occur in some patients. [13] However, CNVs found in autistic individuals are not exclusively observed in autism- they also occur in patients of other neurological disorders. [7]

Single nucleotide polymorphisms

In the following overview, a summary of the SNP analysis in autism till date based on a literature search is presented. The genes that have been studied and details on SNPs follow.

Table 1
Genes studied for association with autism [14]

Chromosome	Genes studied
1	MTHFR, SFN, MTF1, RIMS3, DAB1, PDE4B, CYR61, DPYD, GSTM1, NGF, NOTCH2, RIT1, NTRK1, IFI16, ATP1A2, HSPA6, RFWD2, PTGS2, CHI3L1, MARK1, DISC1
2	SNTG2, NRXN1, RAB11FIPS, NPAS2, DPP10, CNTNAP5, SCN2A, SCN1A, SCN7A, SLC25A12, DLX1, DLX2, RAPGEF4, HOXD11, ITGA4, INPP1, NRP2, PAX3 and CENTG2
3	CNTN4, OXTR, GPX1, CNTN3, ROBO1, ROBO2, DRD3, FBXO40, FXR1, PAK2, DLG1, MBD4, SLC9A9, NLGN1, HTR3C and BCL6
4	DRD5, CD38, SPP1, EIF4E, EGF, PCDH10 and TDO2
5	SEMA5A, CDH10, CDH9, PRLR, DHFR, APC, PITX1, ADRB2, DRD1 and NSD1
6	HLADRB1, PRL, C4B, SYNGAP1, RNF8, GLO1, HTR1B, GluR6, AH11 and PARK2
7	HOXA1, PON1, DLX6, DLX5, TAC1, NPTX2, SERPINE1, RELN, PIK3CG, NRCAM, IMMP2L, DOCK4, FOXP2, MET, FAM4A1, WNT2, CADPS2, UBE2H, NOBOX, CNTNAP2, DPP6 and EN2
8	DLGAP2, MCPH1, SLC18A1, CSMD3, EXT1 and CYP11B1
9	SLC1A1, ASTN2, TSC1 and DBH
10	GATA3, EGR2, TRIP8, REEP3, KCNMA1 and PTEN
11	IFITM3, HRAS, SCT, PTCHD1, BDNF, PAX6, SHANK2, DHCR7, SERPINH1, MTNR1B, PTS, HTR3A, CADM1, ROBO3 and ROBO4
12	AVPR1A, TPH2, IGF1, RFX4, DAO and HSPB8
13	NBEA, HTR2A, DIAPH3 and PCDH9
14	MDGA2
15	TUBGCP5, CYFIP1, NIPA2, NIPA1, NDN, SNRPN, UBE2A, ATP10C, ATP10A, GABRB3 and RORA
16	CACNA1H, TSC2, A2BP1, ABAT, GRIN2A, PRKCB1, SEZ6L2, SLC7A5 and ANKRD11
17	PER1, PAI1, MAP2K3, NOS2A, SLC6A4, NF1, ITGB3, HOXB1, CACNA1G, BZRAP1 and PTDSR
18	MBD1 and BCL2
19	MBD3, GADD45B, TLE6, PDE4A, PLAUR, DMPK and SHANK1
20	OXT and ADA
22	DGCR6, PRODH, TBX1, COMT, MIF, ADORA2A, ADSL and SHANK3
X	ASMT, NLGN4, STS, APIS2, GRPR, CDKL5, PTCHD1, ARX, AL1RAPL1, DMD, MAOA, JARID1C, UPF3B, HOPA, NLGN3, SLC9A6, FMR1, SLC6A8, IRAK1, MECP2, RPL10 and RAB39B
Y	ASMT, NLGN4Y

Chromosome 1

This chromosome contains a susceptibility locus, 1q41-42. Several SNPs in the MARK1 gene within this locus are associated with autism, especially rs12740310, rs3737296 and rs12410279. rs12410279 affects the level of MARK1 gene expression, thus affecting microtubule dependent transport, dendritic transport speed and length. [15]

Hemizygous deletions in DYPD gene on 1p21.3, along with additional mutation in other genes, can lead to autism. [16]

Chromosome 2

AUS5 (autism susceptibility 5) region is located on this chromosome. Different common SNPs in the mitochondrial aspartate/glutamate carrier (SLC25A12) gene on 2q24 are associated with autism. [7] 2p15-16.1 contains genes XPO1 and OTX1 in which rs6735330, rs2018650 and rs13000344 are associated with autism. However, these polymorphisms are not common variants among patients of idiopathic autism and thus contribute in only the rare cases of 2p15-16.1 deletions. [17] 2q31 contains

the gene ITGA4, in which SNPs in exons 16 and 17 are associated with autism. [18] Sodium channel genes SCN1A and SCN2A have SNPs in coding exons and splice sites in autism patients. Variant R1902C in SCN2A reduces the channel's affinity for calcium-bound calmodulin. 38 SNPs have been reported in these genes that need further analysis. [19]

Chromosome 3

Oxytocin plays a role in social cognition and behavior and the oxytocin receptor gene (OXTR) is linked with susceptibility to autism. Several SNP alleles of OXTR occur in autism. [7] 3q29 contains 20 genes (including PAK2, DLG1, FBX045). Microdeletion of 1.6- 2.1 Mb is associated with autism. No SNP analysis studies have been conducted on this. [20] 70 SNPs in 3q25-27 have been studied by linkage analysis, especially those in FXR1 gene, but none contributed to autism in the population studied. [21]

Chromosome 5

5p15 has an SNP between SEMA5A and TAS2R1 genes which has been implicated in autism. [22] 6 SNPs have been found in 5p14.1, between CDH10 and CDH9 (both of which encode neural cell adhesion molecules), of which rs4307059 is the most significant. [23] The region 5q31, containing the PITX1 gene has also been shown to have polymorphisms present in autistic individuals. [24]

Chromosome 6

3 studies have shown association of different SNPs in the Glutamate receptor 6 (GluR6) genes. [7]

Chromosome 7

Most genes assessed in autism are located on 7q22-36 (AUS1 region). [7] Three studies support the involvement of a trinucleotide repeat polymorphism in the 5′UTR region in RELN gene in autism, whereas five other similar studies do not. [7] Different, possibly functional variants in LAMB1 have been associated with autism in two studies. LAMB1's protein promotes neuronal migration and neurite outgrowth during development. [7] Two SNPs in the Engrailed 2 (*EN2*)-gene, a homeobox transcription factor on 7q36, involved in the development of brainstem and cerebellum, are associated with autism in several cases. [7] Common and also rare variants in the contactin-associated protein-like 2 (*CNTNAP2*), a member of the neurexin super family can also increase the risk for autism in certain cases. CNTNAP2 plays a role in the language circuitry, thus being implicated in autism. [7] The gene encoding the MET receptor tyrosine kinase is involved in brain development and gastrointestinal repair. Since some autistic individuals concurrently suffer from gastrointestinal symptoms, a SNP genotyping of this gene has been conducted. A functional promotor variant, several other SNPs as well as rare mutations have been found to be associated with autism in several samples. [7]

Chromosome 8 In MCPH1, CNVs have been found as mentioned earlier. [14]

Chromosome 10

PTEN (phosphatase and tensin homologue, on 10q23.3) is a tumour suppressor gene which is a negative regulator in the PI3K pathway. Heterozygous PTEN mutations have been found in some autistic individuals, resulting in low PTEN levels. [7]

Chromosome 11

In a Korean study, 2 SNPs – rs11212733 and rs7125479 have been found that could be associated with autism [25] while in a Finnish population, 11p12-p13 region (containing glutamate transporter genes)'s SNPs have been found to have no significant association with autism [26].

Chromosome 12

No SNPs from this chromosome have been implicated in autism, but submicroscopic chromosomal anomalies have been found. [14]

Chromosome 15

Several variants of genes on 15q11-13 have been assessed, as cytogenetic abnormalities of this region are observed in autism, but ATP10C, UBE3A and the gamma-aminobutyric acid (GABA) receptor genes located on 15q have given inconclusive findings. [7]

Chromosome 17

Due to presence of platelet hyperserotonemia in autistic children and their first-degree relatives, common polymorphisms in the serotonin-transporter gene (SLC6A4) have been assessed in several studies. [7]

Chromosome 22

Several case reports indicate 22q13.3 deletions and duplications as risk factors for autism. SHANK3 gene, located in this region, encoding a synaptic scaffolding protein, has been assessed for SNPs possibly associated with autism. Several studies have shown both sporadic and inherited mutations in this gene in autistic individuals. Similar to mutations of X-chromosomal genes, SHANK3 mutations might cause a monogenic form of language delay. The frequency of SHANK3 mutations is 0.5–1% in autistic individuals. Common variants have not been detected as risk factors. A recent study, however, reported SHANK3 deletions in healthy individuals, implying that it might be a harmless polymorphism. [7]

X-chromosome

Keeping in mind the skewed sex distribution ratio in autism (males affected four times more than females), several variants in genes on X chromosome have been analyzed for association with autism. Two neuroligin genes-NLGN2 and NLGN4, involved in synapse formation, on Xq13 and Xp22 have been screened for mutations. Despite the discovery of several non-conservative mutations in single families in these genes, the findings could not be replicated in larger samples sizes. Similarly, two mutations identified in the ribosomal protein gene RPL10 on Xq28 could not be replicated in a later study. [7]

Y-chromosome

Few studies have looked into association between variants on the Y chromosome and autism. A recent study has reported negligible association between an SNP in NLGN4Y gene, which had earlier been thought to be associated with autism. Hence, no SNPs on Y chromosome have been associated with autism. [7]

METHOLOGY OF SNP ANALYSIS

In silico methods

Computational SNP analysis is the most convenient and rapid method of SNP analysis but being theoretical, it can merely be used for prediction. Still, the advent of SNP analysis software has made SNP genotyping much simpler now than it was a decade ago, helping to minimize the amount of experimental work and cost. The list of SNPs in a particular gene can be obtained from several databases-dbSNP of NCBI, HapMart of HaploView and HGMD (Human Gene Mutation Database) [27, 47]. Analysis of these SNPs in order to prioritize them in terms of risk potential can be done by numerous tools that are either downloadable or available online. SIFT- Sorting Intolerant From Tolerant- It is a tool developed by the J Craig Venter Institute that assigns risk potential to non synonymous SNPs (nsSNP) based on the type of amino acid change that is occurring. For example, a change from a positively charged amino acid to another positively charged amino acid will not be considered risky whereas a change from polar to non-polar amino acid will be considered very risky. [28- 33] PolyPhen- Polymorphism Phenotyping- It uses physical and evolutionary comparative considerations to predict whether the amino acid change due to a nsSNP will affect the structure and function of human proteins. [34] PANTHER- Protein Analysis Through Evolutionary Relationships- The cSNP tool of PANTHER is based on alignment of evolutionarily similar sequences in order to predict which among a set of non-synonymous SNPs is likely to have deleterious functional

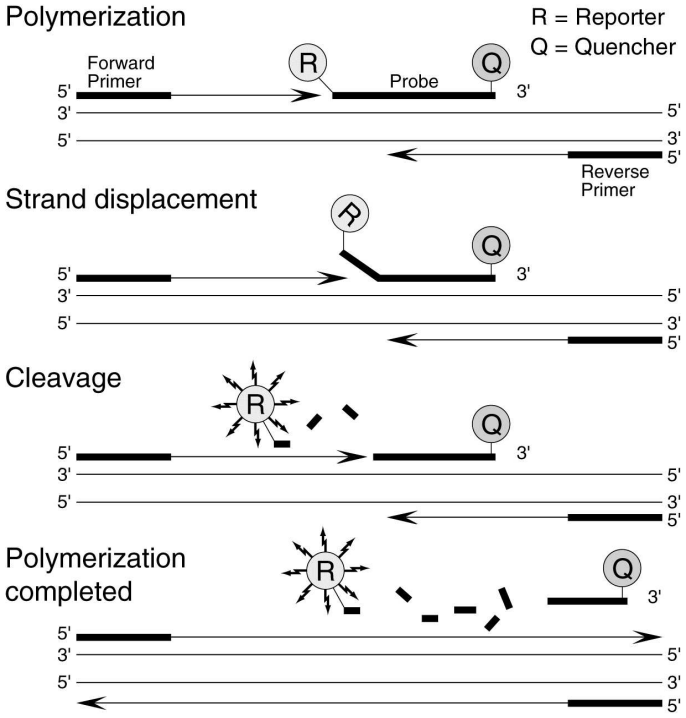
effect on the encoded protein. [35, 36, 37]

FASTSNP- It is based on hidden Markov models and prioritizes SNPs from "Very low risk" to "Very high risk" based on twelve phenotypic risks and putative functional effects. [38] Pupasuite- is an interactive web-based tool selects relevant SNPs within a gene based on characteristics of the SNP (validation status, type, frequency/population data and putative functional properties). It also performs LD plotting on genotype data from HapMap. [39] SNPStats- Its a software package for the analysis of genetic-epidemiology studies of association using SNPs. It can be used for arriving at- allele and genotype frequencies, test for Hardy-Weinberg equilibrium, and analysis of association with a response variable based on linear or logistic regression, multiple inheritance models and analysis of interactions. For multiple SNPs linkage disequilibrium estimation, haplotype frequency estimation, analysis of association of haplotypes with the response and analysis of interactions (haplotypes-covariate) can be performed. [40] Linkage disequilibrium- Usually during SNP analysis, a large set of SNPs needs to be analyzed. LD plotting helps to reduce this number to only those SNPs that are closely associated with the gene of interest. Several tools are available for this- WGAViewer, SNPStats, HaploView and SNP & Variation Suite by Golden Helix. [27, 41] Genome browsing- Genome browser tools like Ensembl and UCSC Genome Browser can be used to determine the location of the SNP to be analyzed on the chromosome. They can be used to determine whether the SNP falls within a protein coding region, promoter sequence, untranslated region, intronic sequence etc. [42, 43]There are numerous other tools and databases for computational analysis of SNPs, with specific uses, advantages and disadvantages.

Experimental methods

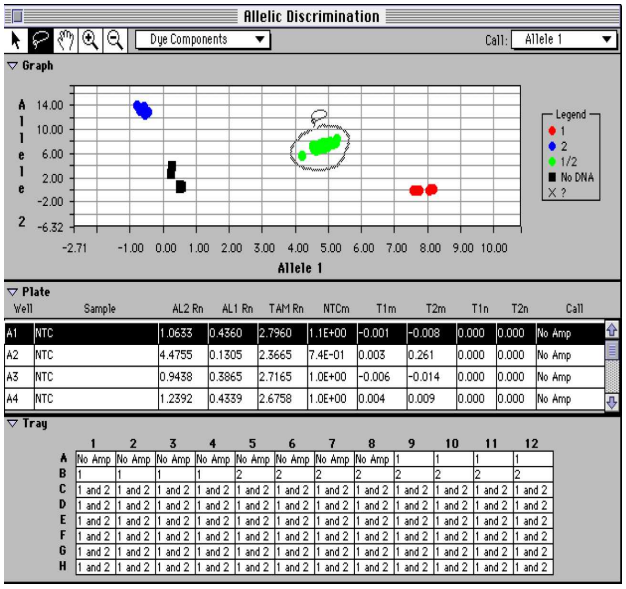
RFLP- This technique is based on the sequence specificity of restriction endonucleases. If a polymorphism is present within this sequence for a particular sample, the enzyme will not cleave it, resulting in an agarose gel electrophoresis band pattern different from the expected pattern. Thus, genotyping can be easily achieved. [44] ARMS-PCR (ARMS= amplification refractory mutation system) - This method detects known point mutations. An ARMS-PCR experiment consists of two reactions- one containing a normal DNA sequence-specific ARMS primer and the other containing a mutant-specific primer. The genotype is determined by analysis of the amplification products: for homozygous normal individual, amplification occurs only for the first reaction, for homozygous mutant individual, amplification occurs only in the second reaction and for heterozygous individuals, reaction will occur in both. [46] A comparative study shows that ARMS-PCR is better. ARMS-PCR method involves a single step PCR. Instead, RFLP-PCR method is laborious, needing more optimization steps, reagents and human handling. [44] ARMS-PCR is suitable for analysis of large number of samples, a situation which occurs often in SNP analysis. [44] Allelic discrimination real time PCR- It uses the 5'- 3' nuclease activity of AmpliTaq Gold DNA polymerase. By PCR, a fluorescent reporter probe attached to the DNA is released, allowing direct detection of PCR product. [45] Two TaqMan probes are used, one for each allele in a two-allele system. Each probe is made of an oligonucleotide with a 5´-reporter dye and a 3´-quencher dye. Differently coloured reporter dyes are used for the two alleles. [45] When the probe is intact, the quencher dye quenches the reporter dye's fluorescence. During PCR, forward and reverse primers hybridize to a specific sequence of the target DNA. The probe hybridizes to a target sequence within the PCR product. The enzyme cleaves the probe with its 5´–3´ nuclease activity as it moves along the strand. Thus, the reporter dye and quencher dye are separated, resulting in fluorescence of the reporter. The 3´ end of the probe is blocked to prevent extension of the probe during PCR. [45]

Figure 1
Principle of allelic discrimination real time PCR [45]



This process occurs in every cycle, without affecting the exponential product accumulation. The separation of the reporter dyes from the quencher dye results in increase in fluorescence for each of the reporters, which is due to target amplification during PCR. [45]

Figure 2
Representative output of allelic discrimination real time PCR [45]



Red fluorescence implies homozygosity for allele 1, blue implies homozygosity for allele 2 and green implies heterozygosity. [45] Both primer and probe must hybridize to their targets for amplification and cleavage to occur. The fluorescence signals are generated only if the target sequences for the probes are amplified during PCR. Because of this, non-specific amplification is not detected. [45] Sequencing- Direct sequencing of DNA by chain termination method is usually the final step in SNP genotyping. Earlier methods involved autoradiography and 4 reaction tubes. The advent of automated sequencers has simplified this to a single reaction tube and automated capillary electrophoresis. With this method, 96 samples can be sequenced at a time. However, for the large number of samples used nowadays, even this is not sufficient. This has led to the development of next-generation high-throughput sequencing technology. This is particularly useful for genotyping applications since only a small fragment has to be sequenced. One of these methods is pyrosequencing. This is based on transformation of the pyrophosphate molecules released during DNA elongation into light (the reaction being PPi to ATP which in turn is utilized by luciferase to emit light). Hence, a real time quantitative determination of nucleotide incorporation is possible. Pyrosequencing has been automated for several platforms and thus is ideal for use in SNP genotyping but its high cost impedes widespread use. [46] Methods based on physical properties of DNA such as denaturing gradient gel electrophoresis, temperature gradient gel electrophoresis, denaturing HPLC, single strand conformation polymorphism and high resolution melt analysis can also be used. However, optimization of the conditions in order to standardize the experiments is rather difficult. In conclusion, numerous methods, both computational and experimental are available for SNP genotyping but choosing the right method poses a challenge to scientists as several factors need to be taken into consideration such as- accuracy, reliability, cost effectiveness and ease of use.

CONCLUSION

Hundreds of SNPs and other genetic markers have been genotyped in several populations of autistic individuals. However, very few studies have been conducted on Indians. We propose that SNP genotyping be done on Indian autistic individuals. This data can find applications in diagnostics, pharmacogenomics and gene therapy. Currently, no specific test is available to diagnose autism and clinicians base their diagnosis on behavior evaluations and questionnaires [48]. Knowing the genetic markers would thus help in accurate, rapid diagnosis in the future.

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