Cell Mol Neurobiol
DOI 10.1007/s10571-014-0038-9

ORIGINAL RESEARCH

Potential Contribution of Dopaminergic Gene Variants in ADHD Core Traits and Co-Morbidity: A Study on Eastern Indian Probands

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Received: 16 October 2013 / Accepted: 20 February 2014

Abstract Association of dopaminergic genes, mainly receptors and transporters, with Attention Deficit Hyperactivity Disorder (ADHD) has been investigated throughout the world due to the importance of dopamine (DA) in various physiological functions including attention, cognition and motor activity, traits. However, till date, etiology of ADHD remains unknown. We explored association of functional variants in the DA receptor 2 (rs1799732 and rs6278), receptor 4 (exon 3 VNTR and rs914655), and transporter (rs28363170 and rs3836790) with hyperactivity, cognitive deficit, and co-morbid disorders in eastern Indian probands. Diagnostic and Statistical Manual for Mental Disorders-IV was followed for recruitment of nuclear families with ADHD probands ($N = 160$) and ethnically matched controls ($N = 160$). Cognitive deficit and hyperactive traits were measured using Conner's parents/teachers rating scale. Peripheral blood was collected after obtaining informed written consent and used for genomic DNA isolation. Genetic polymorphisms were analyzed by PCR-based methods followed by population- as well as family-based statistical analyses. Association between genotypes and cognitive/hyperactivity traits and co-morbidities was analyzed by the Multifactor dimensionality reduction (MDR) software. Case–control analysis showed statistically significant difference for rs6278 and rs28363170 ($P = 0.004$ and 1.332e−007 respectively) while family-based analysis exhibited preferential paternal transmission of rs28363170 '9R' allele ($P = 0.04$). MDR analyses revealed independent effects of rs1799732, rs6278, rs914655, and rs3836790 in ADHD. Significant independent effects of different sites on cognitive/hyperactivity traits and co-morbid disorders were also noticed. It can be summarized from the present investigation that these gene variants may influence cognitive/hyperactive traits, thereby affecting the disease etiology and associated co-morbid features.

Keywords ADHD · Dopaminergic gene variants · Phenotypes · Co-morbidities

Introduction

Attention Deficit Hyperactivity Disorder (ADHD) is an early onset neurobehavioral disorder characterized essentially by inattention, hyperactivity, and impulsivity (Biederman and Faraone 2005; Polanczyk et al. 2007). This developmental disorder has a high worldwide pooled prevalence rate (Polanczyk et al. 2007) where genetic and environmental factors interplay with each other (Biederman and Faraone 2005); estimated heritability for ADHD varies within 60–90 %. As evidenced from twin studies, genetic factors exert major influence on the familial risk (Thapar et al. 1999), while environment may also have significant effect in the etiology (Banerjee et al. 2007).

Beside the triad of externalizing behaviors, co-morbid disorders like learning disability (LD), mood disorder (MD), oppositional defiant disorder (ODD), conduct

Electronic supplementary material The online version of this article (doi:10.1007/s10571-014-0038-9) contains supplementary material, which is available to authorized users.

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Published online: 02 March 2014

disorder (CD), and anxiety disorder (AD) are also often present in ADHD children (APA 1994). Converging evidences point toward deregulations of the pre-frontal cortex (PFC), corpus striatum, and cerebellar circuits to be cause of the disorder (Martin et al. 2002). Major cognitive processes like attention, logical reasoning, verbal fluency, personality development, etc., are driven by the PFC itself (De Young et al. 2010), while cellular atrophy in the medial PFC is related to difficulty in memory reconsolidation (Mander et al. 2013). Due to numerous interconnections with different parts of the brain involved in the cognitive processes (Goldman-Rakic et al. 1990) and being connected with the limbic system, PFC is also associated with emotion and mood changes (Price 1999). PFC receives impulses from the ventral tegmental area (VTA) through the meso-cortical pathway and influences cognitive functions like motivation (Wise 2004), reinforcement learning (Kehagia et al. 2010), working memory (Sawaguchi and Goldman-Rakic 1991; Williams and Goldman-Rakic 1995), etc. ADHD probands have shown performance deficit on tasks assessing frontal cortex functions, such as response inhibition, selective attention, and set sifting (Cornish et al. 2005) making role of dopamine (DA) vital in the disease etiology.

Striatum, as a major component of the nigro-striatal pathway, also plays a vital role in regulating the cortical activity; striatum excites cells of the limbic system through nucleus accumbens (NA) which produces a greater indirect effect on the frontal cortex through substantia nigra and thalamus via the thalamo-cortical circuitry. It has been shown that cellular abnormality in the striatal region is associated with poor executive functioning (Voytek and Knight 2010). A strong correlation between dopaminergic dysfunction in the fronto-striatal area and behavioral outcomes of ADHD patients has also been evidenced through imaging studies (Biederman and Faraone 2005; Arnsten 2006). Moreover pharmacotherapy with methylphenidate (MPH), a blocker of principally DA reuptake, normalizes symptoms to a great extent in about 70 % of children by improving motor and executive functions (Wilens 2008). MPH has been documented to regulate gene expression in the cortico-striatal region (Yano and Steiner 2007) and to induce longer dendritic spine formation in the NA and striatum along with increased FOS B expression in experimental animals (Kim et al. 2009).

Bioavailability of DA in the PFC and striatum is chiefly regulated by the DA type II receptor 2 (DRD2), receptor 4 (DRD4), and DA transporter (DAT), respectively (Kellendonk et al. 2006; Fusar-Poli et al. 2012). *DRD2* and *DRD4*, as well as DAT1 (encoded by *SLC6A3*) have shown strong association with ADHD (Holmboe et al. 2010; Wu et al. 2012). We have analyzed functional variants in these three genes in a group of Indo-Caucasoid ADHD probands in order to find out association of gene variants with ADHD-related cognitive impairment, hyperactivity, and co-morbid features like ODD, LD, MD, and CD.

Materials and Methods

Participants

From the out-patient department of Manovikas Kendra Rehabilitation and Research Institute for the Handicapped, Kolkata, ADHD probands ($N = 160$) were recruited based on the DSM-IV criteria (APA 1994). Hyperactivity level and cognitive attributes were measured by the Conners' Parents and Teachers Rating (CPRS-R) Scale (Conners et al. 1998). Intelligence/developmental quotient were assessed by the Wechsler's Intelligence Scale for children (Wechsler 1991) for children above 5 years and Developmental Screening Test for children below 5 years (Bharat Raj 1971). Mean age of probands was 7.7 years $\pm$ 2.3 SD and male to female ratio was 11.3:1. Out of the 160 probands, 132 were complete parent-proband trios, 16 had only one parent, and 12 were affected probands only. Majority of the probands belonged to the combined subtype (72.5 %), while hyperactive/impulsive (12.5 %) and inattentive (15 %) subtypes were only few. Around 60 % probands exhibited cognitive deficit, while 63 % were hyperactive. Co-morbid conditions were assessed using the DSM-IV criteria for each disorder (APA 1994) and eastern Indian ADHD probands were found to have ODD (35 %), LD (29 %), CD (11 %), MD (11 %), and AD (5 %) as co-morbidity.

A control group ($N = 160$; mean age 19.7 years $\pm$ 7.94 SD; male to female ratio 1.4:1), evaluated following the DSM-IV criteria for ADHD (APA 1994) was also recruited. Control subjects were also assessed for hypothyroidism, intelligence/developmental quotient (>80), as well as for any psychiatric disorder running in the family. Those assessed to have no evidence of anomaly were recruited as healthy control individuals. All the individuals enlisted for the study belonged to the Indo-Caucasoid ethnic category. Informed written consent for participation in the study was obtained from guardians of ADHD probands and controls. The protocol was approved by the Institutional Human Ethical Committee.

Exclusion Criteria

Probands suffering from psychiatric problems including pervasive developmental disorders, any form of mental retardation (IQ $\leq$ 70) and fragile-X syndrome, were excluded.

Table 1 Details on sites analyzed in the current study

Gene	ID	Position in the genome	Allelic variance	Name of the information site/cross reference	Predicted function
DRD2	rs1799732	5′ Promoter region	C/Deletion	Arinami et al. (1997)	Transcriptional regulation
	r6278	3′ Untranslated region	G/T	F-SNP	Splice site regulation
DRD4	VNTR	Exon 3	≤4R/>4 R	(a) Asghari et al. (1995) (b) Schoots and Van Tol (2003)	Higher repeats confer (a) blunted response to DA and (b) reduced receptor expression
	rs916455	5′ Upstream	C/T	F-SNP	Transcriptional regulation
SLC6A3	rs28363170	3′ Untranslated region	40 bp tandem repeats (R) Common variants 9 R/10 R	Brookes et al. (2006)	Transcriptional regulation, tagging marker for polymorphic site
	rs3836790	Intron 8	30 bp tandem repeats (R) Common variants 5R/6R	Hill et al. (2010)	Tagging marker for polymorphic sites

Assessment of Traits

ADHD-associated traits in the probands, i.e., hyperactivity, cognitive impairment, and ADHD index, were classified into four categories based on the CPRS-R score ranging between 0 (T score 38–61), 1 (T score 62–70), 2 (T score 71–80), and 3 (T score 81–90 and above).

Selection of SNPs and Genotyping

Based on prior association reports on ADHD probands from different countries (Guan et al. 2009; Das et al. 2011; Das Bhowmik et al. 2013; Li et al. 2013), six polymorphic sites in three genes, i.e., *DRD2* (rs1799732 and rs6278), *DRD4* (rs916455 and exon 3 VNTR), and *SLC6A3* (rs3836790 and rs28363170) were selected. As detailed in Table 1, functional role of these sites was obtained from published literature or analyzed by F-SNP (http://compbio.cs.queensu.ca/F-SNP/) in the present study.

Peripheral blood was collected from ADHD probands, their parents and controls for isolation of genomic DNA (Miller et al. 1998). Details of oligonucleotide sequences and amplification protocol are provided in Table S1 (Supplementary material).

Data Analysis

Population- and Family-Based Analysis

We have analyzed the genotypic data by both population- as well as family-based association methods. For comparing the allelic and genotypic frequencies, online r x c Contingency Test was used. Genotype counts obtained were analyzed for Hardy–Weinberg Equilibrium (HWE) by online software (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl-hwe). The program COCAPHASE, which is part of a suite of programs UNPHASED (Dudbridge 2003) was used for population-based analysis; allele/genotype frequencies of the control individuals were compared with that obtained for the ADHD proband and their parents. Haplotype-based Haplotype Relative Risk (HHRR) analysis, also under the program UNPHASED, was used for analyzing transmission of markers; transmission from informative, as well as non-informative parents is taken into account for HHRR (Terwilliger and Ott 1992). Comparisons were tested for multiple corrections while running the UNPHASED (1,000 itinerations). The Piface' version 1.72 was used for calculating power of the test showing significant results (Lenth 2007). Odds Ratio Calculator was used to calculate the odds ratio (OR) and its confidence interval (www.hutchon.net/ConfidOR.htm); OR portray the strength of association between two binary data values compared symmetrically. Linkage Disequilibrium between marker pairs in each gene was calculated by the Haploview software (Barrett et al. 2005).

Epistatic Interaction Analysis

Gene–gene interaction analysis of the case–control dataset was performed by the Multifactor Dimensionality Reduction (MDR) program, a data mining strategy which detects and characterizes high-order nonlinear interactions among discrete attributes by a 4-step process, thereby predicting a discrete outcome (Moore et al. 2006).

Initially, noisy polymorphisms were removed from the pool of possible candidates using the Tuned ReliefF (TuRF) filter algorithm (Moore and White 2007) to reduce the chance of overfitting the data which is possible when considering high-order interactions (>4) in relatively small data sets (Moore and White 2007).

Second, by the MDR kernel, a new multilocus attribute for each dimension was created by pooling multilocus genotypes into 1 variable consisting of 2 risk groups (high and low risk). Since number of affected and unaffected

individuals was not equal, balanced accuracy with random seed 1 was used to avoid spurious results due to chance divisions of the data (Ritchie et al. 2001).

Then, a naive Bayes classifier in the context of a 10-fold cross-validation was used to estimate the testing accuracy of each one-dimensional attribute of the 2-factor to 10-factor models. The cross-validation consistency (CVC) was also calculated which measures the number of times out of 10 divisions of the data that the same best model was found (Hahn et al. 2003). The model with a CVC > 5 out of 10, maximum Testing Balanced Accuracy (TBA), and a minimum Prediction Error (PE) for that comparison was considered as the best model (Hahn et al. 2003). Statistical significance (P-values) was evaluated using a 1000-fold permutation test to compare observed testing accuracies with those expected under the null hypothesis of no association; best models were chosen at the 0.05 % significance level.

In the last step, measures of interaction information were used to provide a statistical interpretation of the gene–gene interaction model (Moore et al. 2006). Nature of the dependencies or interactions using the MDR algorithm was determined on the basis of interaction graphs. The MDR interaction model describes percentage of entropy by each factor (information gain or IG). All these analyses were implemented in the open-source MDR software package version 2.0 beta 8.4.

Analysis of Correlation Between Genotypes and Endophenotypes

T score for ADHD index, obtained from the CPRS-R, was plotted against scores for cognitive deficit and hyperactivity. Comparative analysis between genotype frequency observed in ADHD probands and ADHD index score, as well as co-morbid characters was also carried out. While interaction between genotypes and endophenotypes like hyperactivity and cognitive deficit was analyzed by Analysis of variance (ANOVA), association between genotypes and co-morbid behaviors was calculated by r × c contingency test. P values were generated using the tailed T test (http://studentsttest.com).

Result

Genotypes of the *DRD4* exon 3 VNTR deviated from the HWE ($P = 0.001$ in probands and 0.04 in father) which could be due to the difference in frequencies of the higher repeat (R) variants (Table 2). Marginal deviation ($P = 0.03$) was also noticed for rs28363170 for probands and their mother (Table 2) which could be due to higher occurrence of the "9R" allele in these two groups as compared to the control. Other studied sites followed the equilibrium (Table 2).

Allelic case–control comparison (Table 3) revealed statistically significant higher frequencies of rs6278 "G" allele in the father of probands ($P = 0.004$, power 81 %). rs28363170 "9R" allele showed significant higher occurrence in the ADHD probands ($P = 1.332e{-}007$, OR = 3.99, power 99 %), as well as their father ($P = 4.565e{-}029$, OR = 8.55, power 100 %) and mother ($P = 2.381e{-}007$, OR = 3.58, power 99 %) as compared to the control population (Table 3). We have also noticed higher occurrence of the rs3836790 5R allele in ADHD probands ($P = 0.04$, OR = 1.47, power = 52 %) and their father ($P = 0.0004$, OR = 1.94, power = 94 %).

Comparative analysis of genotype frequencies (Table 2) revealed statistically significant higher occurrence of rs6278 "GG" ($P = 0.008$; power = 80 %) and rs3836790 "5R5R" ($P = 0.02$; power = 69 %) in the father group only, while rs28363170 genotypes containing the "9R" allele was higher in families with ADHD probands ($P < 0.05$, power > 50 %).

Family-based analysis (Table S2 in Supplementary material) exhibited no obvious bias in transmission of alleles excepting for rs28363170 which showed a trend for the "9R" allele ($P = 0.09$). Stratified analysis based on gender of the parents revealed that this trend was principally due to paternal bias in transmission of the "9R" allele (T: NT = 18:10; $P = 0.04$, power 53 %).

Comparative analysis on haplotypic frequencies revealed that *SLC6A3* haplotypes harboring the "9R" allele occurred at higher frequencies in ADHD probands and their mothers (Table S3 in Supplementary material; $P = 0.01$ and 0.02, respectively, power 79 % for both).

The marker pairs studied were not in linkage disequilibrium as evidenced from low D' and r^2 values (Table S4 in Supplementary material), and therefore, may not be transmitted together.

ADHD index was found to be high in 48 % of the probands followed by those with low (28 %), very high (15 %), and very low (9 %) ADHD index (Fig. 1). As compared to probands with very low values for cognitive deficit and hyperactivity traits (Fig. 2), statistically significant higher values were noticed in probands with low ($P = 0.001$ and 0.05), high ($P = 5.96e{-}9$ and 0.0005), and very high ($P = 2.73e{-}12$ and 0.0003) scores for these traits, respectively. Probands with very high ADHD index also exhibited higher scores for cognitive deficit, as well as hyperactivity (Fig. 2).

Stratified analysis between genotype frequency and ADHD index (Table 4; Supplementary Fig. 1) revealed higher frequencies of the rs1799732 "Del" variant in probands with higher index as compared to those with very

Table 2 Genotypic frequency observed in control individuals ($N = 120$), ADHD probands ($N = 160$), their father ($N = 154$), and mother ($N = 143$)

Gene	ID	Genotype	Control	*P* value for HWE	ADHD cases	*P* value for HWE	Chi square (*P*)	Father of ADHD cases	*P* value for HWE	Chi square (*P*)	Mother of ADHD cases	*P* value for HWE	Chi square (*P*)
DRD2	rs1799732	CC	0.76	0.06	0.68	0.18	1.6 (0.450)	0.75	0.14	0.245 (0.885)	0.69	1.0	1.23 (0.54)
		CDel	0.20		0.27			0.22			0.26		
		DelDel	0.04		0.05			0.29			0.49		
	rs6278	GG	0.44	0.30	0.50	1.0	2.65 (0.26)	0.61	1.0	**9.59 (0.008)**	0.46	0.31	3.36 (0.186)
		GT	0.41		0.42			0.35			0.47		
		TT	0.15		0.08			0.04			0.07		
DRD4	Exon 3	≤4R	0.91	1.00	0.85	0.001	5.26 (0.07)	0.87	0.04	1.52 (0.46)	0.86	0.23	2.22 (0.33)
		≤4R > 4R	0.09		0.10			0.12			0.14		
		>4R > 4R	0.00		0.05			0.01			0.01		
	rs916455	CC	0.89	1.0	0.88	0.18	1.01 (0.605)	0.87	1.0	0.19 (0.67)	0.91	0.08	1.50 (0.47)
		CT	0.11		0.11			0.13			0.08		
		TT	0.0		0.01			0.0			0.01		
SLC6A3	rs28363170	9R/9R	0.01	0.08	0.07	0.03	**17.1 (0.0001)**	0.03	1.0	**5.93 (0.052)**	0.08	0.03	**17.3 (0.0001)**
		9R/10R	0.10		0.28			0.21			0.27		
		10R/10R	0.89		0.64			0.76			0.64		
	rs3836790	5R/5R	0.04	0.77	0.08	0.68	3.06 (0.22)	0.12	0.06	**7.70 (0.02)**	0.06	0.67	2.36 (0.31)
		5R/6R	0.31		0.38			0.48			0.38		
		6R/6R	0.65		0.54			0.46			0.55		

Statistically significant differences are presented in bold

Table 3 Allelic frequency of control individuals ($N = 120$), ADHD probands ($N = 160$), their father ($N = 154$), and mother ($N = 143$)

Gene	ID	Allele	Control	Probands	Chi square (P)	Odd's ratio (95 % CI)	Father of ADHD cases	Chi square (P)	Odd's ratio (95 % CI)	Mother of ADHD cases	Chi square (P)	Odd's ratio (95 % CI)
DRD2	rs1799732	C	0.86	0.82	1.34 (0.25)	–	0.85	0.062 (0.80)	–	0.83	0.61 (0.43)	–
		Del	0.14	0.18			0.15			0.17		
	rs6278	G	0.64	0.71	2.048 (0.15)	–	0.78	**7.98 (0.004)**	0.51 (0.27–0.94)	0.70	1.834 (0.176)	–
		T	0.36	0.29			0.22			0.30		
DRD4	Exon 3	≤4R	0.955	0.925	2.134 (0.144)	–	0.925	2.05 (0.15)	–	0.930	1.559 (0.21)	–
		≥4R	0.044	0.074			0.074			0.069		
	rs916455	C	0.974	0.94	0.22 (0.63)	–	0.93	0.33 (0.57)	–	0.95	0.03 (0.86)	–
		T	0.052	0.06			0.07			0.05		
SLC6A3	rs28363170	9R	0.06	0.21	**27.82 (1.332e−007)**	**3.995** (1.77–9.00)	0.49	**125.2 (4.565e−029)**	**8.55** (4.6–15.8)	0.21	**27.08 (1.95e−007)**	**3.589** (1.59–8.06)
		10R	00.94	0.79			0.51			0.79		
	rs3836790	5R	0.20	0.27	**4.04 (0.04)**	**1.47** (0.76–2.82)	0.33	**12.32 (0.0004)**	**1.94** (1.03–3.63)	0.26	5.229 (0.07)	1.40 (0.72–2.70)
		6R	0.80	0.73			0.67			0.74		

Statistically significant differences are presented in bold

low index ($P < 0.005$ for low, high, and very high index groups). Significant association of rs6278 "GG" with very high ADHD index was also observed ($P = 0.001$). DRD4 exon 3 VNTR > 4 repeats were found to be associated with higher ADHD index ($P > 0.001$, power > 85 %). rs916455 "CC" and rs3836790 lower repeat variants also showed a trend for association with high ADHD index ($P = 0.06$ and 0.04, respectively); however, the power was low. ANOVA analysis for testing of association between individual genotype and phenotypic attributes revealed significant association of the DRD4 exon 3 higher repeats with high scores for hyperactivity (Table 5), while rs3836790 "5R" showed a trend for association with cognitive deficit.

Analysis of case–control data by MDR exhibited independent effects of rs1799732, rs6278, rs916455, and rs3836790 in ADHD probands (Fig. 3a). rs1799732 and rs3836790 along with DRD4 exon 3 and rs28363170 also showed independent effects on cognitive deficit (Fig. 3b), while rs1799732, rs914655, and rs3836790 had effects on hyperactivity (Fig. 3c). Further analysis revealed risk of association for rs1799732, rs6278, and rs916455 in ADHD probands (Table S5 in Supplementary material; $P < 0.01$; power = 100 %). Significant correlation of cognitive deficit and hyperactivity with DRD4 exon 3 and SLC6A3 variants was also noticed (Table S5 in Supplementary material; $P < 0.001$; power = 100 %).

ADHD + LD probands showed statistically significant higher frequencies of rs1799732 "CC" and rs916455 "CC" genotypes ($P = 0.009$, power = 73 %; $P = 0.02$, power = 64 %, respectively) as compared to the control individuals (Table 6). On the other hand, rs1799732 "DelDel" genotype was higher in ADHD + ODD probands ($P = 0.008$; power = 75 %; Table 6). rs6278 "GG" ($P > 0.0001$; power = 95 %) and rs916455 "TT" ($P > 0.01$; power = 70 %) genotypes were higher in ADHD + CD probands while the DRD4 exon 3 higher repeats were noticed in probands with co-morbid LD and CD ($P < 0.005$). rs28363170 "9R" was found to be associated with all co-morbid conditions ($P > 0.0001$, power > 90 %). ADHD + MD probands revealed higher frequencies of genotypes with rs3836790 "5R." The rs28363170 "9R" and rs3836790 "5R" also showed higher occurrence in ADHD-co-morbidity (Table 6; Supplementary Fig 2).

Significant independent effects of rs1799732, rs6278, rs914655, and rs3836790 on co-morbid disorders were also evident from MDR analysis (Fig. 4a–d). Additionally interactive effects between rs1799732–rs3836790, rs6278–rs3836790 was noticed in LD, ODD, as well as MD (Fig. 4a, b and c, respectively), while rs6278-rs28363170 also had effect on LD/ODD (Fig. 4a, c). Two way analysis also confirmed significant contribution of the studied sites

on ADHD-associated co-morbidities where effect of the *SLC6A3* variants were more pronounced (Table S6 in Supplementary material; $P < 0.01$; power = 100 %).

Discussion

Since the dopaminergic system has an impact on the frontal cortex, as well as subcortical regions like striatum, areas of the brain reported to be affected in ADHD probands (Holmboe et al. 2010), we selected genes having regulatory function on these regions. Our objective was to find out association of six functional dopaminergic gene polymorphisms with ADHD-associated cognitive deficit, hyperactivity, and co-morbid characteristics. Data obtained is indicative of significant independent effects of these sites on ADHD core traits with independent, as well as interactive effects on the co-morbid conditions.

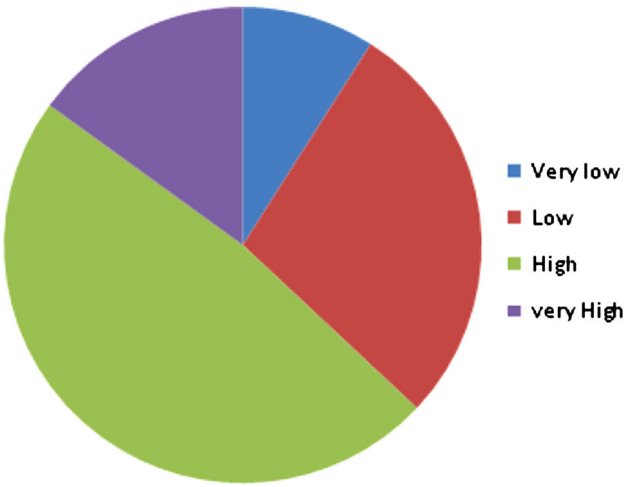

Fig. 1 Frequency of probands exhibiting different levels of ADHD index

Dopamine Receptors 2 and 4 (*DRD2* and *DRD4*)

Both DRD2 and DRD4 are integral to the plasma membrane and aids in binding of DA, drug, and proteins. On the other hand, while DRD2 controls growth and locomotor behavior, DRD4 regulates DA metabolism and feedback. DRD2 receptor has a higher density in the striatum than the cerebral cortex (Ito et al. 1999) and is the principal target for pharmacological intervention of several neuropsychiatric and neurologic disorders (Al-Eitan et al. 2012). Association of *DRD2* SNPs with ADHD has been studied in probands from Finland (Nyman et al. 2007), Brazil (Zhang et al. 2006), as well as Spain (Parsons et al. 2007). rs1799732 Del variant was reported to alter transcriptional activity of the promoter, thus changing receptor expression (Arinami et al. 1997). This variant was also found to influence D2 receptor density in the striatum (Arranz and de Leon 2007), as well as response to antipsychotic drugs (Zhang et al. 2010). F-SNP analysis in the present study revealed that rs6278, at the 3′UTR, regulate splicing of the transcript. Earlier investigators have mainly explored association of neuropsychiatric disorders with rs1799732 (Kraschewski et al. 2009; Filbey et al. 2012) and rs6278 (Bergen et al. 2005; Huang et al. 2009). We first attempted to find out contribution of these sites in Indo-Caucasoid ADHD probands and though our initial attempt ($N = 152$) failed to show any allelic bias for both the sites (Das Bhowmik et al. 2013), a follow up study ($N = 170$) revealed nominal bias for the rs1799732 "C" along with statistically significant occurrence and maternal over transmission in the ADHD + LD group (Ghosh et al. 2013). The present study revealed that higher number of individuals harboring the rs1799732 "CC" genotype also

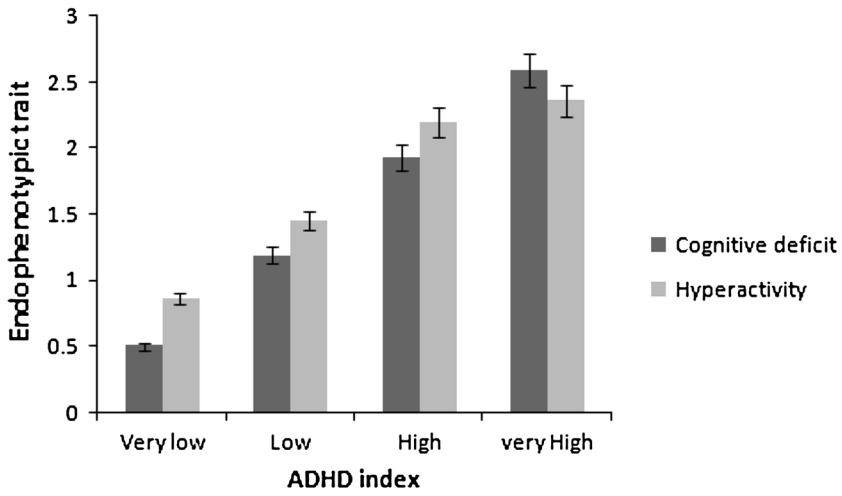

Fig. 2 Correlation of ADHD index with cognitive deficit and hyperactive traits

Table 4 Association of genotypes with ADHD index ($N = 160$)

Gene	ID	Genotype	Very low ADHD index	Low ADHD index	Chi square (P)	Power (95 % CI)	High ADHD index	Chi square (P)	Power (95 % CI)	Very high ADHD index	Chi square (P)	Power (95 % CI)
DRD2	rs1799732	CC	0.86	0.59	**21.8 (0.0001)**	98	0.68	**10.3 (0.006)**	77	0.63	**19.2 (0.0001)**	97
		CDel	0.14	0.30			0.29			0.24		
		DelDel	0	0.11			0.03			0.13		
	rs6278	GG	0.42	0.44	0.123 (0.94)	–	0.48	1.76 (0.41)	–	0.62	**14.4 (0.001)**	90
		GT	0.50	0.49			0.41			0.24		
		TT	0.08	0.07			0.11			0.14		
DRD4	Exon 3	≤4R	100	0.83	**18.6 (0.0001)**	96	0.87	**13.9 (0.001)**	89	0.66	**41.0 (0.0001)**	100
		≤4R > 4R	0.0	0.12			0.09			0.24		
		>4R > 4R	0.0	0.05			0.04			0.10		
	rs916455	CC	0.83	0.81	0.14 (0.71)	–	0.88	5.61 (0.061)	49	0.80	0.30 (0.58)	–
		CT	0.17	0.19			0.09			0.20		
		TT	0	0.0			0.03			0		
SLC6A3	rs28363170	9R/9R	0.07	0.07	3.06 (0.21)	–	0.10	0.83 (0.66)	63	0.10	5.01 (0.08)	32
		9R/10R	0.29	0.34			0.25			0.32		
		10R/10R	0.64	0.59			0.65			0.58		
	rs3836790	5R/5R	0.15	0.07	4.08 (0.13)	–	0.07	**6.32 (0.043)**	40	0.20	0.88 (0.64)	–
		5R/6R	0.31	0.28			0.46			0.30		
		6R/6R	0.54	0.65			0.47			0.50		

Statistically significant P values are presented in bold

Table 5 Analysis of association between genotypes and phenotypic attributes of ADHD probands ($N = 160$)

Gene	ID	Genotypes	Cognitive deficit	Hyperactivity/ Impulsivity
DRD2	rs1799732	CC	–	–
		CDel		
	rs6278	GG	–	–
		GT		
DRD4	DRD4 exon 3	≤4R > 4R	–	0.02
		>4R > 4R		
	rs916455	CC	–	–
		CT		
SLC6A3	rs28363170	9R9R	–	–
		9R10R		
	rs3836790	5R5R	0.08	–
		5R6R		

had very low ADHD index as compared to other ADHD individuals, while those harboring the rs6278 "GG" were found to have very high ADHD index. rs1799732 "C" and rs6278 "G" were more frequent in ADHD + LD and ADHD + CD, respectively, while those with co-morbid ODD had higher frequency of the rs1799732 "Del" allele. rs1799732 also showed independent effect on cognitive deficit, as well as hyperactivity, and thus may affect the etiology as a whole. Independent effects of rs1799732 and rs6278 in the co-morbid groups also support the earlier report that *DRD2* may contribute to co-morbid conditions (Ghosh et al. 2013). Moreover, rs6278 showed interactive effects with *SLC6A3* sites in these co-morbid groups, more specifically in ADHD + LD, ADHD + MD, and ADHD + ODD. We may infer from the present study that both rs1799732 and rs6278 play role in the etiology of ADHD-associated co-morbidities and pharmacological intervention targeting the DRD2 receptor could be useful for addressing the neurophysiological phenomena altered in ADHD probands with various co-morbidities.

ADHD probands are believed to have an altered function of the frontal lobe (Lazar and Frank 1998) and DRD4, with a higher number of receptors in this region, obviously becomes a target to study the disease etiology. A role of DRD4 also stems from a number of positive association studies carried out throughout the world (Kereszturi et al. 2007; Lasky-Su et al. 2010; Banaschewski et al. 2010; Das et al. 2011; Das Bhowmik et al. 2013). A 48 bp tandem repeat in the third cytoplasmic loop of the receptor was studied by a number of investigators and the higher repeat variant (7R), reducing sensitivity to DA (Asghari et al. 1995; Schoots and van Tol 2003), was reported to confer risk of ADHD (Lasky-Su et al. 2010; Gizer et al. 2009; Banaschewski et al. 2010). Association of higher repeats has also been observed in the Indo-caucasoid ADHD probands (Das Bhowmik et al. 2013). The present study showed nominal increase in frequency of the higher repeat alleles in ADHD probands, as well as their parents as compared to the control, though the difference was statistically insignificant. Further, association of lower repeats with very low ADHD index was observed, while presence of even a single higher repeat was associated with hyperactivity thus affecting the ADHD index and co-morbid CD and LD. On the other hand, higher frequency of the lower repeats was observed in probands without any co-morbidity or co-morbid ODD and MD. MDR analysis revealed that this site has significant contribution on cognitive deficit in this population. In healthy Caucasian adults from the Netherlands, *DRD4* 7R carriers exhibited significantly higher scores on self-reported dysfunctional impulsivity that involves tendency to act with less forethought than the most people with equal ability (Colzato et al. 2010). The 7R has also been associated with faster habituation in infancy and increased novelty seeking in adults (Laucht et al. 2006) while imposing sensation seeking in toddlers (Sheese et al. 2007) and impulsivity, as well as lower levels of response inhibition in healthy adults (Congdon et al. 2008). Association of the 7R allele with poorer performance in CPT was also noticed while those homozygous for the 4R demonstrated better performance (Kieling et al. 2006). Individuals harboring even one 7R allele showed slower reaction time performance with a more pronounced deficit in males (Szekely et al. 2011). The exon 3 VNTR was speculated to cause underlying executive dysfunction (Langley et al. 2004). However, in the Caucasian population from US, individuals harboring the 7R allele were found to have better cognitive performance (Gornick et al. 2007) and children with the 7R allele made fewer errors on a test of sustained attention than those without the allele (Bellgrove et al. 2005b; Johnsson et al. 2008). Healthy individuals of Spain homozygous for the 7R allele, displayed higher accuracy for the go/no-go task as compared to those homozygous for the 4R allele (Kramer et al. 2009). An association of the 4R with less efficient executive functioning in healthy populations from US and China (Fossella et al. 2002) was also reported. In ADHD probands, the 4R allele showed significant association with symptoms of inattention (Lasky-Su et al. 2010) and increased ADHD symptomatology (Bidwell et al. 2011). Earlier investigators have also shown association of higher repeats with ADHD associated conduct problems (Holmes et al. 2002; Gabriela et al. 2009). Whether this observed difference in association of the repeats is due to gross difference in allelic frequencies in different populations or is due to the procedure employed in estimation of association merits further in depth investigation.

Functional analysis of rs916455 by F-SNP revealed that this substitution may result in altered transcriptional

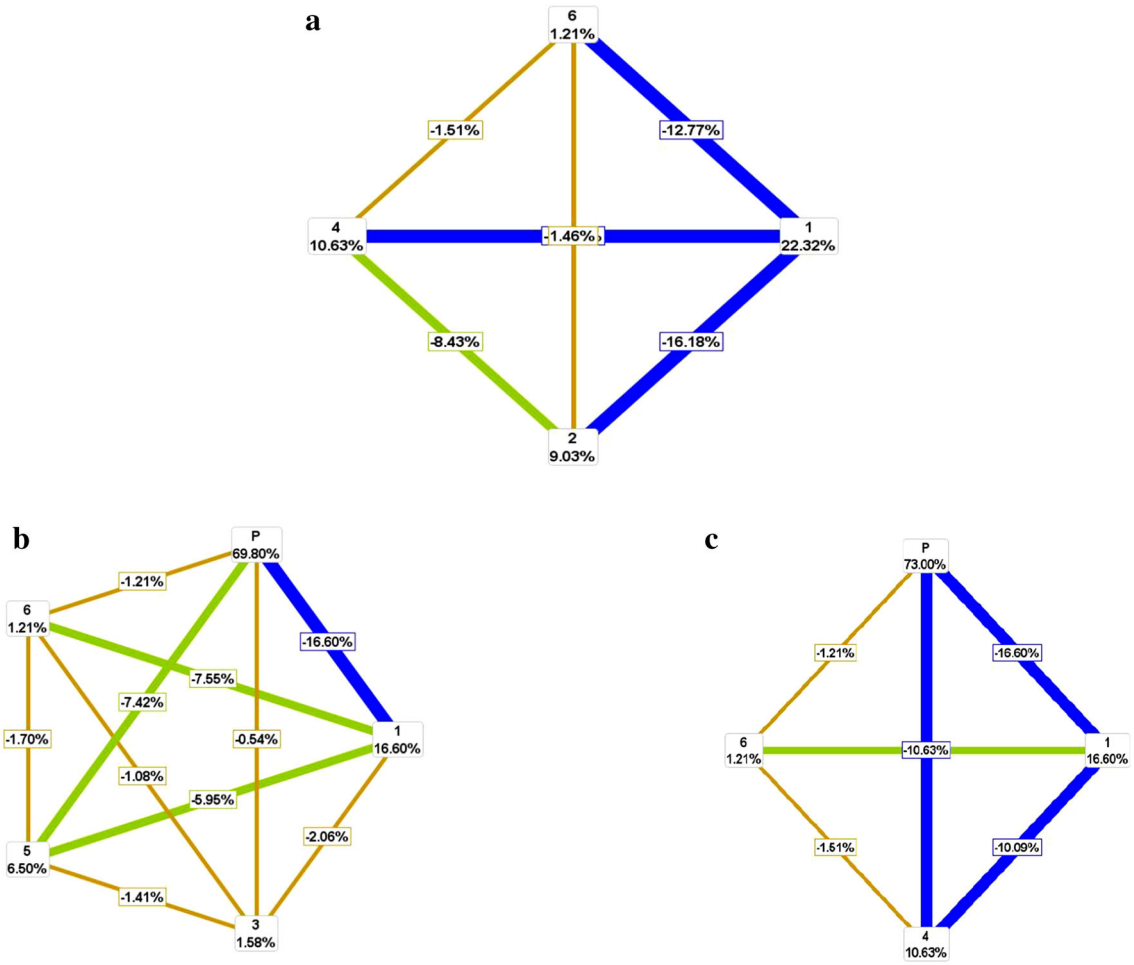

Fig. 3 Gene–gene interaction analyzed using case–control data in ADHD cases (**a**), considering cognitive deficit (**b**) and hyperactivity (**c**) as endophenotypes. All the positive IG values in the nodes indicate independent main effect of all the markers. All the *lines* with negative IG values indicate redundancy or lack of any synergistic interaction between the markers. Nodal No. 1—DRD2 rs1799732, 2—DRD2 rs6278, 3—DRD4 ex 3, 4—DRD4 rs914655, 5—SLC6A3 rs28363170, 6—SLC6A3 rs3836790, P—endophenotypes (cognitive deficit/hyperactivity)

regulation. Earlier investigations in Finnish (Nyman et al. 2007) and Korean (Yang et al. 2008) ADHD probands failed to show any significant contribution of rs916455. In the Indo-Caucasoid probands also, this site failed to show any significant difference in occurrence of allelic/genotypic frequencies as compared to the control population, as well as parents of probands. However, stratified analysis revealed a trend for association of the "CC" genotype with higher ADHD index and ADHD + LD, while the ADHD + CD probands showed higher frequency of the "TT." MDR analysis showed independent effect of this SNP on ADHD, hyperactivity and all four co-morbid conditions. The "TT" genotype was detected at a very low frequency in ADHD probands and their mother, whereas none of the control individuals showed this genotype. In the Chinese population, "C" allele carriers were predicted to have more persistent ADHD symptoms (Li et al. 2013). To find out the actual role of this site, further exhaustive functional analysis is warranted in extended number of samples since the "T" allele frequency is extremely low in the population.

Dopamine Transporter (*DAT1/SLC6A3*)

In the present investigation, analysis of rs28363170 in the 3′UTR revealed statistically significant higher occurrence of the "9R" allele ($P > 0.001$) and genotypes ($P > 0.001$), as well as haplotypes ($P = 0.01$) containing this allele with a paternal bias in transmission ($P = 0.04$). Further, an independent effect of this site was observed on higher ADHD index and cognitive deficit. Association analysis with co-morbidities showed that the presence of the "9R" allele in ADHD probnads is associated with ODD, LD, CD, as well as MD. We have also noticed significant association

Table 6 Difference in genotypic frequencies in ADHD-co-morbidity and various co-morbid groups

Gene	ID	Genotype	Control	ADHD-co-morbidity	Chi square (*P*)	ADHD + ODD	Chi square (*P*)	ADHD + LD	Chi square (*P*)	ADHD + CD	Chi square (*P*)	ADHD + MD	Chi square (*P*)
DRD2	rs1799732	CC	0.76	0.72	5.44 (0.07)	0.55	**9.77 (0.008)**	0.91	**9.52 (0.009)**	0.83	1.36 (0.51)	0.71	0.65 (0.72)
		CDel	0.20	0.28		0.38		0.09		0.13		0.24	
		DelDel	0.04	0		0.07		0		0.04		0.05	
	rs6278	GG	0.44	0.44	2.68 (0.27)	0.55	2.47 (0.30)	0.55	3.56 (0.17)	0.72	**17.3 (0.0001)**	0.40	0.74 (0.69)
		GT	0.41	0.48		0.32		0.37		0.17		0.47	
		TT	0.15	0.08		0.13		0.08		0.11		0.13	
DRD4	Exon 3	≤4R	0.91	0.96	2.06 (0.15)	0.89	0.22 (0.64)	0.66	**20.2 (0.0001)**	0.87	**11.0 (0.004)**	0.88	0.47 (0.48)
		≤4R > 4R	0.09	0.04		0.11		0.26		0.04		0.12	
		>4R > 4R	0.00	0		0		0.08		0.09		0	
	rs916455	CC	0.89	0.89	0.0	0.83	1.50 (0.22)	0.94	**7.71 (0.02)**	0.75	**8.81 (0.01)**	0.88	0.49e−01 (0.83)
		CT	0.11	0.11		0.17		0.03		0.20		0.12	
		TT	0.0	0.0		0		0.03		0.05		0	
SLC6A3	rs28363170	9R/9R	0.01	0.07	**18.9 (0.0001)**	0.04	**17.4 (0.0001)**	0.12	**16.3 (0.0001)**	0.09	**22.8 (0.0001)**	0.12	**18.2 (0.0001)**
		9R/10R	0.10	0.30		0.32		0.21		0.31		0.23	
		10R/10R	0.89	0.63		0.64		0.67		0.60		0.65	
	rs3836790	5R/5R	0.04	0.11	**7.21 (0.03)**	0.04	0.57 (0.75)	0.10	2.79 (0.25)	0.04	0.23e−1 (0.99)	0.12	**10.3 (0.006)**
		5R/6R	0.31	0.41		0.36		0.30		0.32		0.44	
		6R/6R	0.65	0.48		0.60		0.60		0.64		0.44	

Significant *P* values are presented in bold

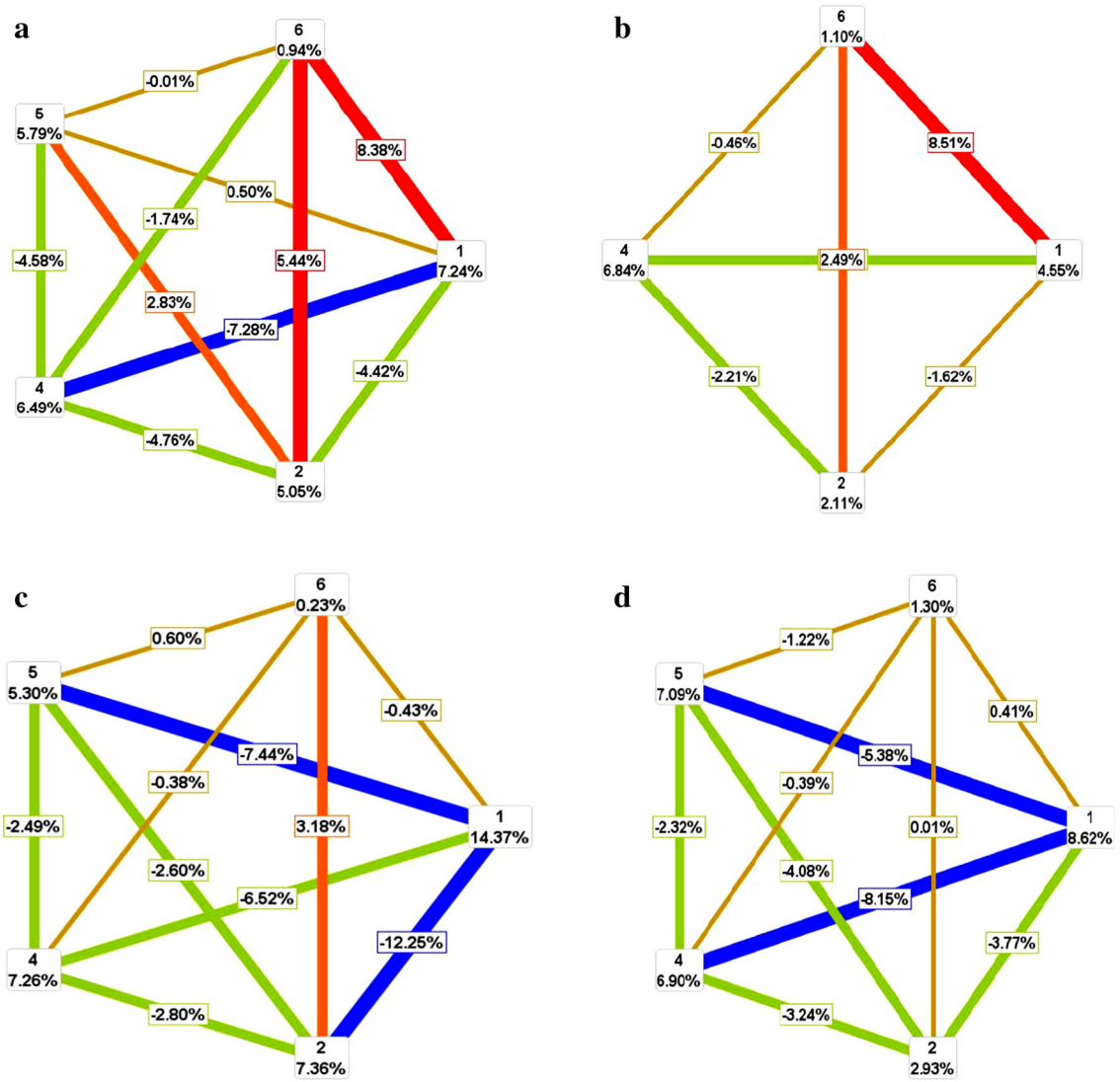

Fig. 4 Gene–gene interaction analyzed in ADHD cases exhibiting co-morbid **a** LD, **b** MD, **c** ODD, and **d** CD using case–control data. All the positive IG values in the nodes indicate independent main effect of all the markers. All the *lines* with negative IG values indicate redundancy or lack of any synergistic interaction between the markers. Nodal No. 1—DRD2 rs1799732, 2—DRD2 rs6278, 3—DRD4 ex 3, 4—DRD4 rs914655, 5—SLC6A3 rs28363170, and 6—SLC6A3 rs3836790

of the rs3836790 (intron 8 VNTR) "5R" allele with high ADHD index. A strong interactive effect of rs3836790 with DRD2 rs6278 was noticed by MDR analysis in ADHD subjects with co-morbid LD (nodal value 5.44), MD (nodal value 2.49), and ODD (nodal value 3.18). rs1799732 exhibited interaction with rs3836790 in co-morbid LD and MD (nodal values 8.38 an 8.51 respectively). The 3′UTR 9R allele was earlier reported to be associated with adult ADHD (Franke et al. 2010) and increased task-related default-mode network suppression (Brown et al. 2011). Presence of at least one copy of the 9R allele was correlated with higher transporter density presumably leading to effective DA clearance from the synapse (van Dyck et al. 2005; van de Giessen et al. 2008). The rs3836790 repeats were also reported to act differentially (Hill et al. 2010). The 10R-6R haplotype formed between the 3′UTR-int 8 VNTR and the 10R/10R genotype of the 3′UTR was suggested as risk factors for ADHD in children (Brookes et al. 2006; Asherson et al. 2007; Faraone et al. 2005); 10R/10R homozygous children performed poorly on a sustained attention task as compared to others (Loo et al. 2003; Bellgrove et al. 2005a). Neuroimaging studies in adults showed more efficient neural response in the pre-frontal cortex of individuals homozygous for the 10R during working memory task (Bertolino et al. 2006; Caldú et al. 2007). Contradictory to these, presence of the 9R showed association with higher impulsivity (Forbes et al. 2009). Association between poorer response inhibition and

homozygosity for the 10R allele was also noticed (Cornish et al. 2005). DAT1 was inferred to have a more significant role in hyperactivity than inattention (Diamond 2007) and associated only with ADHD in children without co-morbid CD (Zhou et al. 2008). It was suggested that DAT1 has a modulatory role rather than a causative role (Franke et al. 2010). In non-clinical subjects, individuals carrier for the DRD4 7R and homozygous for the DAT1 10R showed impaired inhibition on the stop-signal task relative to other genotype groups (Congdon et al. 2008). Greater neural activation was also reported for carriers of the 9R allele (Congdon et al. 2009). Another study revealed greater neural activation in the left striatum, right dorsal premotor cortex, and right temporoparietal cortical junction during response inhibition in individuals homozygous for the 10R as compared to those harboring at least one 9R allele (Bedard et al. 2009). Further, a high density SNP mapping showed significant association of DAT1 with response inhibition phenotype though the 3′UTR VNTR failed to show any significant contribution (Cummins et al. 2012). From the large number of studies conducted, it is evident that DAT1 has a role in the complex etiology of ADHD. However, further work is warranted to find out the actual mode of action.

Phenotypic alterations in ADHD is speculated to be contributed by changes in dopaminergic signaling (Lazar and Frank 1998; DiMaio et al. 2003; Shaw et al. 2007) and correlation with poor performance in tasks involving the frontal cortex function was documented (Cornish et al. 2005). Gene variants studied in the present investigation were hypothesized earlier to potentially affect attention via frontal subcortical circuits linking the frontal cortex to distinct areas of the striatum (Alexander et al. 1986; Cummings 1993; Nieoullon 2002; Cummings and Miller 2007; DiMartino et al. 2008). Association of dopaminergic gene variants with different phenotypic attributes of Indo-Caucasoid ADHD probands was explored for the first time and significant correlation of the studied sites with cognitive deficit, hyperactivity, as well as co-morbid conditions was noticed. Major limitation of the present investigation is the small number of samples used and further work in extended number of samples is warranted to validate the observed significant association. However, this preliminary study revealed significant contribution of the *DRD2, DRD4*, and *SLC6A3* gene variants in different endophenotypes and could be useful for generating a database for comparative analysis. Primarily differences were obtained by case–control analysis; ADHD probands, as well as their parents showed differences in genotypic frequencies in comparison to the control population which may indicate that families with ADHD probands have a different genetic makeup as compared to the ethnically matched controls, thus providing further support to the "familial risk" of the disorder. In this investigation, we have observed higher transmission, as well as independent effect of *DRD2* rs1799732 "C" allele. We have also noticed higher frequencies for rs28363170 "9R" and rs3836790 "5R" alleles of the *SLC6A3* gene which may cause a delay in clearance of DA from the synapse. rs1799732 was earlier hypothesized to increase DRD2 density (Harrison and Weinberger 2005) and in the murine system, striatal over expression of DRD2 influenced DA levels, DA turnover, and activation of D1 receptors in the PFC, the brain structure mainly associated with working memory (Kellendonk et al. 2006). On the basis of the above observations, we may infer that these two together could alter DA signaling in the eastern Indian ADHD probands. The *DRD4* exon 3 and *SLC6A3* intron 8 VNTR were found to contribute to hyperactivity and cognitive deficit, respectively, while all the variants were associated with co-morbid conditions. Earlier investigators have also documented sharing of a number of gene variants by ADHD probands (Comings et al. 2000). To identify the complex etiology of ADHD, further in depth study on functional attributes is warranted.

Acknowledgments Authors are thankful to the volunteers for participation in the study. Part of this work was sponsored by the Department of Science & Technology, Govt. of India (SR/CSI/17/2009); SM is a DST project fellow. PG (Indian Council of Medical Research, India) and KS (Council of Scientific and Industrial Research, India) received fellowship support from Govt. agencies.

Conflict of interest The authors declare that they have no conflict of interest.

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