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Lack of association of transthyretin variations with spinocerebellar ataxia in north Indian population

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Abstract

Background & Objective: Transthyretin (TTR) has been associated with spinocerebellar ataxia (SCA) by several independent case reports. Coexistence of TTR and SCA mutations, overlapping clinical symptoms as well as altered levels of TTR in SCA patients suggest a correlation between TTR and SCA. To our knowledge, no large cohort based study has been attempted to examine the association of SCA with polymorphism in *TTR* gene. Here, we chose to investigate *TTR* variations in SCA patients (n=266) and controls (n=192) of North Indian ethnicity. *Methods:* We sequenced the exons including exon-intron boundaries of *TTR* gene in 55 patients and 55 controls. We observed four variations which were further genotyped by single base extension method (SNaPshot) in a larger cohort (SCA patients n=211 and controls n=137). *Results:* A novel synonymous variation c.372 C>G in exon 4 was detected in heterozygous condition in one control sample. We found nominal association for rs1800458 (Gly6Ser), with SCA (p-value < 0.05) which did not remain after Bonferroni correction for multiple tests. Pairwise linkage disequilibrium (LD) analysis revealed no LD between studied SNPs. Further, we employed two-marker sliding window analysis and observed a weak association of haplotype AT of rs1800458 and rs1667251 with SCA patients (p-value <0.05) which was not retained after Bonferroni correction.
Conclusion: Our data suggests no association of genetic variations of TTR in SCA pathology.

INTRODUCTION

Transthyretin amyloidosis (ATTR) is the most common form of hereditary systemic amyloidosis. ATTR is a late onset disorder and has been detected in most ethnic groups. More than 100 mutations in transthyretin gene (*TTR*) have been described and majority of these are associated with ATTR. The extensive β-sheet structure of TTR is related to its amyloidogenic potential and it is postulated that most of these mutations stimulate TTR aggregation by destabilizing its native tetramer.[1] In addition, certain mutations have been shown to be non-amyloidogenic in nature.[2] TTR has been linked with various pathological conditions including neurodegenerative disorders.[3-7] Few studies have suggested coexistence of TTR with spinocerebellar ataxia. The TTR mutation Val 30 Met causing Familial amyloid polyneuropathy (FAP) and SCA1 mutation causing central nervous system dysfunction were observed to coexist in a Japanese family.[5,8] Moreover, many independent reports show that cerebellar ataxia is a major clinical feature in patients with leptomeningeal amyloidosis[9-13] and meningocerebrovascular amyloidosis [14,15] associated with different mutations in *TTR*. Recently, decreased levels of transthyretin have been reported in SCA2[16] and SCA12 patients[17] of north Indian ethnicity which further justifies its role in SCA. However, there have been no studies to define the association of SCAs with *TTR* polymorphisms. The above findings stimulated us to carry out a case-control association study in the north Indian population to determine the role of *TTR* polymorphisms in SCA patients.

METHODS

SCA patients (n=266) were recruited from the

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clinical services of All India Institute of Medical Sciences (AIIMS), Delhi. Detailed history of illness and treatment was documented. The cohort for this study includes 103 genetically confirmed unrelated spinocerebellar ataxia patients with identified CAG repeat expansion in 29 SCA1, 10 SCA2, 14 SCA3, 1 SCA7, 47 SCA12 and GAA repeat expansion in 2 FRDA cases. The number of trinucleotide repeats was above the threshold levels for each SCA subtype (SCA1>39, SCA2>32, SCA3>45, SCA7>36, SCA12>51, FRDA>67). One hundred and sixty three genetically uncharacterized SCA patients with unidentified mutations were also included. All selected cases had clinical manifestation of progressive cerebellar ataxia with or without other neurological features. Demographic details of all the patients are provided in Table1. Additionally, 192 unrelated healthy individuals of north Indian origin with no history of SCA and any other neurological disorders were also included. Blood samples from each patient were withdrawn at the time of enrollment.

The study was approved by the Institute Human Ethics Committee of CSIR-Institute of Genomics and Integrative Biology and AIIMS. The study was conducted according to the principles of the Helsinki Declaration. Written informed consent was obtained from all the participants after describing entire study protocol.

Sequencing and Genotyping of TTR polymorphisms

Genomic DNA was extracted from peripheral whole blood using a modified salting-out procedure.[18] All the four exons of *TTR* (NM_000371; NG_009490.1) were sequenced using primers positioned in the intronic regions flanking the exons. Sequencing was carried in both forward and reverse directions on automated genetic analyzer (ABI 3130XL, ABI, Foster City) and sequences were analyzed using SeqMan module of DNAstar tool. Further genotyping was carried out by single base primer extension method (SNaPshot) through capillary electrophoresis in genetic analyzer (ABI 3130xl sequencer). The SNaPshot primers were designed using Massarray software (Sequenom).

Statistical analysis

All the SNPs were tested to ensure their conformance with Hardy-Weinberg equilibrium. Differences in allelic and genotypic frequencies were compared in cases and controls using Fisher's Exact Test and Chi-square test. Bonferroni correction was applied to provide experiment wide significance (for n independent tests significance level α adjusted to $\alpha_{(n)} = \alpha / n$). The unphased data was phased through PHASE tool using parameter values of 100 iterations, a thinning interval of 10, and a burn-in value of 100 in the MCMC simulations.[19] Further, to examine the combined effect of alleles, haplotypes were constructed through two marker sliding window using PLINK software.[20] LD pattern for all the four studied SNPs was measured by calculating D' values using Haploview program (version 4.2).[21]

Expression analysis of the wild type and variants of TTR

TTR cDNA was amplified from pDONR vector (Invitrogen Cat#.12535035) and cloned in a TA vector pTZ57R/T (Fermentas InsTAclone PCR cloning kit). TTR cDNA was subsequently subcloned at *EcoRI and SacII* sites in pEGFP-N3 mammalian expression vector. The TTR variants Gly6Ser (rs1800458), Arg104Arg (c.372 C>G,

Table 1: General clinical demographic of the spinocerebellar ataxia patients

Parameters	Mutation positive SCAs(SCA1, SCA2, SCA3, SCA12 and FRDA)	Mutation Negative SCAs (genetically uncharacterized)
Total No. Of patients	80	136
Gender (Male/Female)	60/20	98/38
Age at examination (mean±SD), range in years	42.7 ± 13.3 (18-75)	38.8 ± 33.6 (4-87)
Age at onset (mean±SD), range in years	37.3 ± 13.3 (15-74)	33.6 ± 18.9 (1-86)
Familial/sporadic cases	62/18	48/88

Detailed clinical records were available for only 216 patients

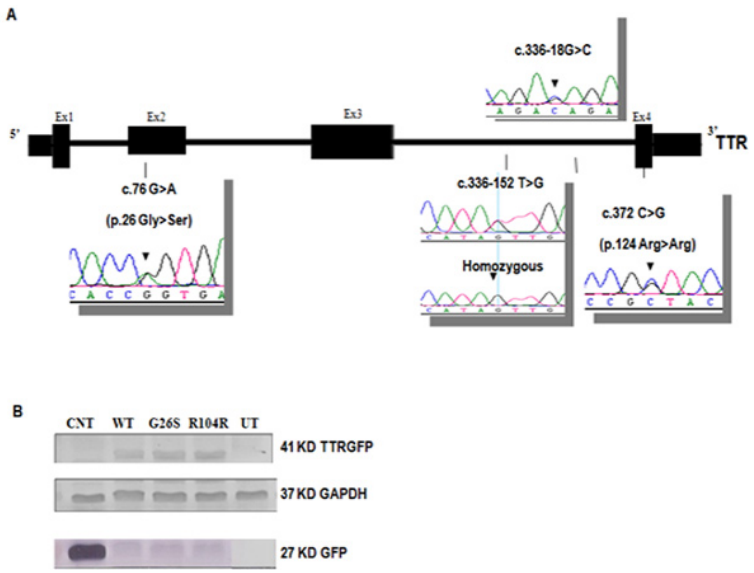

Figure 1 (A). Schematic representation of the four *TTR* variations identified in SCA patients. (B). The immunoblot shows the expression levels of wild type and variants of TTR in HEK293 cell line (Lane1: vector control, lane2: wild type, lane3: rs1800458 (Gly26Ser), lane4: c.372C>G (Arg124Arg), lane5: untransfected control. GAPDH was used as an endogenous control.

novel variation) were created (QuickChange II site directed mutagenesis kit, Stratagene) and confirmed by sequencing.

The human embryonic kidney cell line HEK293 obtained from National Centre for Cell Science, PUNE, was verified for the lack of endogenous expression of TTR as reported earlier[22]. Transient transfections were done using lipofectamine-2000 (Invitrogen Cat.11668019), cells were harvested after 48 hours of incubation and lysed. The total protein from the cell lysate was normalized and resolved on 12% SDS-PAGE. The gel was immunoblotted and probed using GFP antibody (Sigma Cat.G6795). GAPDH (sigma) was used as an endogenous control.

RESULTS

TTR variations and haplotyping in spinocerebellar ataxia patients

The coding region of the *TTR* gene including exon-intron boundaries was sequenced in 55 SCA patients of different subtypes and 55 controls of same ethnicity. We detected four different variations (Figure 1A). A previously reported non-synonymous variation, rs1800458 which causes Gly6Ser change in exon 2 of *TTR* was identified in SCA patients but not in control samples. Two previously reported intronic variations present upstream to exon4: rs1667251 and rs36204272 were also observed. In addition, a novel variation c.372 C>G in exon 4 was detected. This novel variation results in a synonymous Arg to Arg change at position 104 of the protein sequence. All the variations were typed in a larger cohort of 211 patients and 137 controls. The two intronic variations (rs1667251 and rs36204272) did not display any significant association among cases and controls. The novel synonymous (pArg104Arg) variation was observed in heterozygous condition in one control individual but not in SCA patients. No variations were observed in exon 1 and exon 3 of *TTR* gene.

The non-synonymous rs1800458 variation was observed in 10 out of 266 SCA patients where 3 of 29 SCA1, 1 of 2 Friedreich's Ataxia (FRDA), 1 of 47 SCA12, 5 of 163 uncharacterized SCA patients were positive. None of the SCA2, SCA3 and SCA7 patients had this variation. One of 192 controls was positive for this variation. An allele frequency of 0.019 in total SCA patients and 0.003 in control subjects was obtained which indicates weak association (p=0.03). However, the significance was lost after correction for multiple tests. Allelic and genotypic frequencies and p-values for studied SNPs are described in Table 2. Moreover allelic frequencies observed in our population were almost similar to other global populations. However, distribution of alleles (rs1667251) in African population was drastically different from other populations.

Table 2: Frequency distribution of alleles and genotypes of the four markers (rs1800458, rs1667251, rs36204272 and c.372 C>G) of *TTR* gene with spinocerebellar ataxia.

SNP	Region	Chromo-some position	Sample	Allele distribution		Genotype distribution			p-value[c]	
				1	2	11	12	22	Allele	Genotype
rs1800458 (a=G, b=A)	Exon 2	27426863	SCA	0.981 (522)	0.019 (10)	0.962 (256)	0.038 (10)	0 (0)	**0.0303**	**0.0255**
			Controls	0.997 (383)	0.003 (1)	0.995 (191)	0.005 (1)	0 (0)		
rs1667251 (a=T,b=G)	Intron 3	27432377	SCA	0.936 (498)	0.064 (34)	0.872 (232)	0.128 (34)	0 (0)	0.7885	0.69
			Controls	0.93 (357)	0.07 (27)	0.859 (165)	0.141 (27)	0 (0)		
rs36204272 (a=G,b=C)	Intron 3	27432511	SCA	0.983 (523)	0.017 (9)	0.966 (257)	0.034 (9)	0 (0)	1.00	0.878
			Controls	0.984 (378)	0.016 (6)	0.969 (186)	0.031 (6)	0 (0)		
c.372 (a=C, b=G)	Exon 4	27432515	SCA	1.00 (532)	0.00 (0)	1 (266)	0 (0)	0 (0)	0.4192	0.239
			Controls	0.997 (383)	0.003 (1)	0.995 (191)	0.005 (1)	0 (0)		

[a]Major allele, [b]Minor allele
[c]Uncorrected p-values

Table 3: Identification of haplotypes in two-marker sliding window at *TTR* locus in north Indian population

SNPs	Haplotype	Haplotype count		p-value
		Cases	Controls	
rs1800458-rs1667251	GT	488	356	Reference
	AT	10	1	**0.0306**
	GG	34	27	0.7891
rs1667251-rs36204272	TG	489	351	Reference
	GG	34	27	0.7884
	TC	9	6	1
rs36204272-c.372G/C	GC	523	377	Reference
	CC	9	6	1
	GG	0	1	0.4195

Pairwise LD analysis revealed no LD between these SNPs in our cohort (Indian) as well as in African, American, European and East Asian cohorts (Figure 2). Further, haplotype analysis of the four variations showed a weak association of AT haplotype between rs1800458 and rs1667251 with SCA patients which did not hold after Bonferroni corrections (Table 3).

As several emerging evidences suggest hampered TTR levels in various neurodegenerative diseases, we analyzed the effect of two exonic variations: rs1800458 and c.372 C>G on TTR levels in human embryonic kidney cell line HEK293 on transient transfection. However, we did not observe any significant change in TTR protein levels on immunoblotting (Figure 1B).

Clinical characteristic of patients positive for rs1800458

All the ten patients' positive for rs1800458 had features of progressive cerebellar ataxia and the age of the patients were 13-48 years with age at onset ranging between 6-46 years (Table 4). Only one patient was a female with a sporadic genetically uncharacterized ataxia. The findings of radiological investigations were available for all except for one patient. Cerebellar atrophy was a consistent feature in all the patients. Two of the three SCA1 patients positive for rs1800458 had documented white matter hyperintensities (WMH) in the cortical areas of brain.

The records of eight available nerve conduction studies (NCS) showed presence of varying degree of neuropathy in six patients both in the characterized SCA mutation and uncharacterized cases. The SCA12 patient positive for rs1800458 had features suggestive of bilateral (B/L) grade3 Carpel Tunnel Syndrome (CTS) with prolonged distal latencies in motor and sensory NCS of B/L median nerves. In the rest of the nerves tested NCS were normal.

DISCUSSION

In the present study we searched for variations in the exon/exon-intron boundaries of *TTR* gene amongst the North Indian SCA patient-control cohort to investigate the possible role of TTR in the pathogenesis of SCA. Our data shows only minor effect of rs1800458, the non synonymous SNP in SCA patients in North Indian cohort which may be attributed to small sample size. The two intronic variations (rs1667251 and rs36204272) exhibited allele frequencies similar to other world populations (Table 5) and did not show any significant association with SCA in Indian population. Several previous studies have failed to detect significant association of TTR SNPs with different neurodegeneration and cerebrovascular disease including schizophrenia[23], mental retardation, lewy body disorders (LBD)[24] and AD.[25,26] The two SNPs identified in our sample set (rs1800458 and rs36204272) did not earlier exhibit association with schizophrenia and LBD.[23,24] Interestingly, a recent study describes association of certain *TTR* SNPs with neuroimaging endophenotypes in AD patients. Five common SNPs in the promoter and intronic region (rs3764479, rs723744, rs1080094, rs3764476 and rs3794884) were reported to be significantly linked in AD patients for bilateral medial temporal atrophy (MTA) in Caucasian families.[7] The non-synonymous rs1800458 variation exhibited significant association for White matter hyperintensities (WMH) in AD

Table 4: Summary of spinocerebellar ataxia patients with rs1800458 variations.

Patient ID	AT0089	AT0304	AT0392	AT0664	AT0736	AT0851	AT1003	AT1004	AT0245	AT0268
SEX	M	M	M	M	M	M	F	M	M	M
Year	1998	2000	2001	2003	2003	2004	2005	2005	2000	2000
AO	15	24	40	37	28	46	6	32	31	24
Age	35	27	46	42	41	48	13	46	36	30
DD	20	3	6	5	13	2	7	14	5	6
Diagnosis	FRDA	SCA1	SCA1	SCA1	SCA12	UN	UN	UN	UN	UN
Mutation	Homozy-gous GAA expansion	ATXN1-CAG repeats; 29/54	ATXN1-CAG repeats; 32/46	ATXN1-CAG repeats; 27/48	PP-P2R2B-CAG repeats; 14/66	Negative	Negative	Negative	Negative	Negative
Symptom at Onset	Writing Difficulty	Limb-Incord	Walking difficulty	Walking difficulty	Walking difficulty	Walking difficulty	Walking difficulty	Walking difficulty	Walking difficulty	Speech Slurring
Cerebellar Ataxia	+	+	+	+	+	+	+	+	+	+
Nystagmus	+	-	-	+	+	+	-	-	+	-
Dementia	-	-	-	+	+		-	-	-	-
DTR	Absent	Brisk	Brisk	Brisk	Brisk	NL	Absent	reduced	-	++
Plantar	⇑ ↑↑	-	-	⇑ ↑	↑ ↑	-	-	-	-	↑ ↑
Inheritance	Aff Sib	AD	SP	SP	AD	SP	SP	SP	SP	SP
MRI/CT*	Mild CA+CBA	*Mild CBA	CBA/T2-WMH	Severe CBA B/L FP WMH	Mild CBA	*Mild CBA	NA	Diffuse CA	CBA	CT-NL, MRI-ND
NCS	SN UL & LL	NL	NL	SMN UL & LL	B/L CTS GRADE-3	ND	NA	SMN UL & LL	SMN	SN

AD;autosomal dominant, SP;sporadic, CA;cerebral atrophy, CBA; cerebellar atrophy, WMH; white matter hyperintensities, SN; sensory neuropathy, SMN; sensorimotor neuropathy, CTS; carpal tunnel syndrome, ND; not done; NA; not available, UL; upper limbs, LL; lower limbs, (+) means presence of features, (-) means absence of features

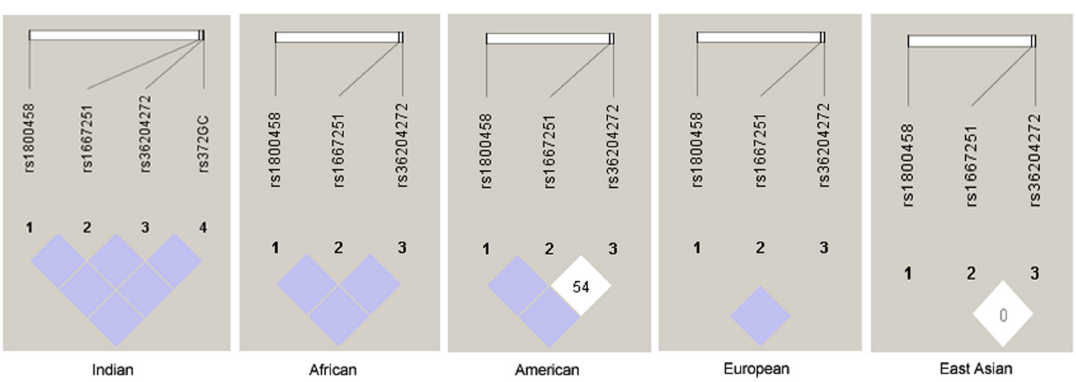

Figure 2. Linkage disequilibrium plot for TTR SNPs for various populations. The figures show the output of the Haploview linkage disequilibrium (LD) plot, where each square represents a pairwise LD relationship between the two single nucleotide polymorphisms (SNPs). Red squares indicate statistically significant LD between the pair of SNP as measured by D' up to a maximum of 1. White squares indicate pairwise D' values less than 1 with no statistically significant evidence of LD. (Each unit of D' shown in multiples of 100).

Table 5: Distribution of allelic frequency of variants in Indian population and across the world.

	rs1800458		rs1667251		rs36204272		Novel SNP	
	minor allele A	major allele G	minor allele G	major allele T	minor allele C	major allele G	minor allele G	major allele C
Cases	0.019	0.981	0.064	0.936	0.017	0.983	0	1
Controls	0.003	0.997	0.07	0.93	0.016	0.984	0.003	0.997
Europe	0.079	0.921	0	1	0.037	0.963	NA	NA
Africa	0.002	0.998	0.252	0.748	0.081	0.919	NA	NA
East Asia	0	1	0.016	0.984	0.054	0.946	NA	NA
America	0.039	0.961	0.08	0.92	0.088	0.912	NA	NA

patients in Caucasian families.[7] Interestingly, two SCA1 patients positive for rs1800458 in our sample set showed WMH. Thus, our work supports the hypothesis that it is important to take neurodegenerative changes into account to establish a genetic correlation of TTR with clinically heterogeneous neurodegenerative disorders including SCA.

Differential TTR levels have been implicated in AD and Parkinson's diseases.[7, 24] In two recent studies, decreased plasma transthyretin levels have been observed in SCA2 and SCA12 patients with sensory neuropathy.[16,17] Unfortunately, transthyretin levels were not available for our SCA patient pool. However, no significant change in the protein levels in HEK293 cell line was observed due to two rs1800458 and the novel synonymous variation. It is well documented that rs1800458 is a non-amyloidogenic variation that results in increased affinity for thyroxine and is associated with euthyroid hyperthyroxinemia.[27] Thus, it is probable that the functional impairment (carrying and transporting thyroxine) due to this variation may be involved in the pathology of spinocerebellar ataxia, however which needs to be further investigated.

In conclusion, we identified four SNPs in *TTR* gene and carried out allele and haplotype based association analysis of these SNPs with SCA patients in North Indian cohort. However, no significant genetic association of TTR with SCA was observed in our patient cohort. It is possible that we failed to detect any correlation due to small sample size. Further investigations in larger sample sets of different ethnicities followed by functional validation may be useful to define the correlation between transthyretin and spinocereberal ataxia.

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DISCLOSURE

Conflict of Interest: None

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