***CFTR* Gene Mutations and Asthma in Indian Children: A Case–Control Study**

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Abstract Cystic Fibrosis Trans membrane conductance regulator (*CFTR*) gene is an asthma susceptibility gene. In the present study we investigated the possible association of *CFTR* gene mutations in Indian asthmatic children as compared to controls. The study included 250 asthmatics and 250 age and sex matched controls. Case to control ratio for sample size was 1:1. Genotyping was performed for 24 *CFTR* gene mutations by ARMS-PCR and PCR–RFLP method. Among 24 *CFTR* gene mutations, heterozygous allele of R553X mutation was found in 4 (1.6 %) asthmatic cases and 2 (0.8 %) controls. Value of FVC and FEV1/FVC ratio were significantly lower in heterozygous individuals (p value <0.05). No significant difference was observed in the genotype and allele frequency of R553X mutation (OR = 1.339, 95 % CI = 0.755–2.374, p value = 0.685). Furthermore, all wild type homozygous alleles were observed in remaining 23 *CFTR* gene mutations. Our data concludes that R553X mutation was not significantly associated in Indian asthmatic children.

Keywords Asthma · *CFTR* gene · North India · Mutations

Introduction

Asthma is a major chronic inflammatory and polygenic disorder. It is associated with symptoms of episodes of progressively worsening shortness of breath, cough, wheezing and chest tightness or a combination of these symptoms [1]. Multiple genetic loci and a variety of environmental factors have been suggested as important determinants for asthma [2–4]. Variations in prevalence of asthma have been found worldwide. Its prevalence ranges from 10 to 17 % in other countries [5]. In Asian countries (2–4 %) low prevalence of asthma has been reported whereas, in United Kingdom, Canada, Australia, New Zealand, and other developed countries (15–20 %) high prevalence of asthma has been reported [6]. In India, the prevalence of asthma in adults is 2.38 % [7] and in children it varies from 6 to 31 % [8].

It has been suggested that more than 100 genes have been found to be associated with asthma in various populations [9] and cystic fibrosis trans-membrane conductance regulator (*CFTR*) gene is one of them located on chromosome 7q31.2. It is a 230 kb gene, encoding a 1,480 amino acid polypeptide, named cystic fibrosis trans-membrane regulator [10]. Functions as chloride channel in the epithelial membrane. It participates in fluid and electrolyte absorption in airway epithelia causes thick, sticky mucus in the lungs, pancreas, and other organs. In the lungs, this mucus blocks the airways, causing lung damage, making it hard to breathe, and leading to serious lung diseases like asthma. In the pancreas, it clogs the pathways leading to

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the digestive system, interfering with proper digestion. In 90 % of cystic fibrosis cases, the airways are affected [11].

CFTR is an asthma susceptibility gene [12]. Carriers of *CFTR* gene mutations (heterozygote) show no symptoms of CF, even though hetero-zygosity has been associated in some studies with male infertility [13] and chronic pancreatitis [14, 15]. Controversial results have been found with respect to asthma and *CFTR* gene mutations that either there is a positive association between mutations and asthma [16, 17] or they protect hetero-zygotes from bronchial asthma [18]. Various association studies have been performed to assess the association of *CFTR* gene with asthma. In context to Asia, a study in 2006, was conducted in Asian (Singaporean Chinese) asthmatic and idiopathic bronchiectasis patients. They reported three missense mutations (1125T, 1556V, and Q13 52H) and 1 splice variant (intron 8 12TG5T) representing a combined mutant/variant allele frequency of 0.25 [19]. Studies have also been conducted in different populations in context with association of the *CFTR* gene and asthma; however, the results have been conflicting with some showing positive association and others showing either protective or no association. A study in 2002 by Joseph et al. represents the frequencies of the *CFTR* gene mutations in cystic fibrosis cases however none of the mutations were reported in India in asthmatic cases yet. Of the various mutations and polymorphisms studied with respect to bronchial asthma, only a few have been found associated with asthma (Δ F508, R75Q, R553X, G576A, L997F, T338M, M1R and V11I) [20].

The spectrum and distribution of CF in Indian asthmatics is still not well known. It is therefore highly desirable to collect this information in order to provide appropriate genetic services. Thus, hypothesis of the present study was that to detect the type and frequency of *CFTR* gene mutation in Indian children. We previously reported the most common *CFTR* gene mutations in our population [21] and in this study we tried to assess the association of other *CFTR* gene mutations in asthmatic children as compared to control population.

Materials and Methods

This was a hospital based case–control study. Included were 250 bronchial asthma cases and 250 age and sex matched healthy controls aged 5 months–15 years, from the indoor and outdoor facility in Department of Pediatrics and Translational Medicine Unit, King George's Medical University Lucknow. Case to control ratio for sample size was 1:1.

Cases and controls were recruited on the basis of inclusion and exclusion criteria. The study was approved by the Institutional Ethics Committee (IEC). Written

informed consent was obtained from the parent/guardian of children before enrolment in the study.

Characterization of phenotype: Cases were characterized on the basis of the presence of following inclusion criteria: (1) current presence of wheeze (2) on any regular medication for asthma (3) currently hospitalized and diagnosed as case of bronchial asthma and (4) presenting with first episode of wheeze along with positive family history of asthma in mother, father, or sibling. Cases excluded were children presenting with clinical symptoms of cystic fibrosis such as meconium ileus at birth, recurrent or persistent pneumonia, mal-absorption causing failure to gain weight and growth problems, salt losing syndromes or having sweat chloride level >60 mmol/L and suffering from other respiratory diseases.

Controls were selected from the same source of population which was homogenous with regard to ethnic background. The inclusion criteria of the controls were age- and sex-matched children with no history of bronchial asthma or any other respiratory disease. And excluded were children presenting with clinical symptoms of CF or having sweat chloride level >60 mmol/L, having a history of allergic disorder or either of whose parents had bronchial asthma.

Variables for Data Collection

Data were collected on age, sex, weight, height, consanguinity, family characteristics, residential area, socio-economic status, immunization status, past history of asthma/allergy, family history of asthma, use of separate cooking space, use of biomass fuels for cooking, smoking status of parents and the presence of an industrial factory in nearby area.

Sweat Chloride Test

Sweat chloride test was performed in 216 cases and 112 controls. For this test, firstly sweat was induced by Webster sweat inducer through pilocarpine iontophoresis. Later after sweat sample was collected in a Macroduct sweat collector (Wescor, Inc.) from each individual for more than 30 min. It was analyzed using a sweat check conductivity analyzer (sweat check 3120). Reference ranges were determined on the basis of the value of chloride level in the sweat: normal (0–40 mmol/L), intermediate (40–60 mmol/L), and abnormal (>60 mmol/L).

Spirometry

Spirometer (Spiro lab II, MIT II, Longfian Scitech Company Boading China) was used to perform lung function

Table 1 Designed primers used for polymerase chain reaction

S.no.	Mutations	Location	Primer sequences (5'–3')	Enzyme	Band size
1	Y122X	Exon 4	N-GAAGGCATAAGCCTATGCCTAAA M-GAAGGCATAAGCCTATGCCTAAT C-ATATTGAAAA TAGTGCTAAG AGTT		N = 273 bp M = 273 bp
2	1078delT	Exon 7	C-CATTTTTTGCAAAGTTCATTAGAA N-GCC TTCTTCTTCT CAGGGTTCTGT M-GCC TTCTTCTTCT CAGGGTTCTGG		N = 274 bp M = 274 bp
3	R347P		F-AGACCATGCTCAGATCTTCCAT R-GCAAAGTTCATTAGAACTGATC	FspI HhaI	N = 258 + 152 M = 410 bp
4	R347H				N = 259 + 151 bp M = 410 bp
5	A455E	Exon 9	F-TAATGGATCATGGGCCATGT R-ACAGTGTTGAATGTGGTGCA	Acil	N = 280 + 278 bp M = 558 bp
6	ΔI507	Exon 10	N-TG GCACCATTA AGAAAATATAA M-TG GCACCATTA AGAAAATATAT C-CATGTGTTTGCAAGCTTCTTAA		N = 275 bp M = 275 bp
7	Q493X		F-GCAGAGTACCTGAAACAGGA R-CATTACAGTAGCTTACCCA	BspCNI	N = 231 + 151 + 109 bp M = 231 + 260 bp
8	V520F			HgaI	N = 17 + 174 bp M = 491 bp
9	R560T	Exon 11	F-CAACTGTGGTTAAAGCAATAGTGT R-GCACAGATTCTGAGTAACCATAAT	HphI	N = 223 + 202 bp M = 425 bp
10	G576A	Exon 12	F-AGCACATAGCATGGTACTTGCCCTC R-TTGAATCCTGGCCAGTAGGGC	BciVI	N = 444 + 220 bp M = 664 bp
11	R668C	Exon 13	F-CAATCCTAACTGAGACCTTACACT F-AATCCTAACTGAGACCTTACCCC R-TGATGCGAGGCAGTATCGCCTCT		N = 279 bp M = 279 bp
12	L997F	Exon 17a	F-GCTATAGCTCCAATCACAATTAATAAC F-GCTATAGCTCCAATCACAATTAATAAG R-GGGAGATATACCTAATGCTAGATGA		N = 281 bp M = 281 bp
13	3659delC	Exon 19	N-CCAACAGAAG GTAAACCTATC M-CCAACAGAAG GTAAACCTATA C-GAGAAGGAAATGTTCTCTAA		N = 117 bp M = 117 bp
14	1898 + 1G → A	Intron 12	N-TAACAGAAAA AGAAATATTT GAAATG M-TAACAGAAAA AGAAATATTT GAAATA C-TTTTAATGGAAGTAATCTTGAATC		N = 117 bp M = 117 bp
15	2183AA → T	Exon 13	N-TGT CTCCTGGACA GAAACAAAACA M-TGT CTCCTGGACA GAAACAAAACG C-CACGCTGATGCGAGGCAGTAT		N = 216 bp M = 216 bp
16	2789 + 5G → A	Intron 14b	N-GCTGTGGCTCCTTGGAAGTGCG M-GCTGTGGCTCCTTGGAAGTGCA C-GCTAGGACTACAGGAATGTGTCA		N = 212 bp M = 212 bp

C common, N normal, M mutant, F forward, R reverse

test on children >6 years of age. The best result of three spirometry maneuvers was selected for the analysis of obstructive disorders and disease severity. Measurements

like forced expiratory volume in one second (FEV1), forced vital capacity (FVC), and FEV1/FVC ratio were included. FEV1 and FVC measurements were expressed as

Table 2 Method of genotyping and annealing temperature of the designed primers

S.no.	Mutation	Method	T^m required ($^{\circ}\text{C}$)
1.	R560T	PCR–RFLP	60
2.	Q493X/V520F	PCR–RFLP	58
3.	3659delC	PCR–RFLP	55
4.	R347H/R347P	PCR–RFLP	58
5.	A455E	PCR–RFLP	57
6.	2183AA $\rightarrow$ T	PCR–RFLP	55
7.	2789+5G $\rightarrow$ A	PCR–RFLP	55
8.	G576A	PCR–RFLP	57
9.	R668C	ARMS–PCR	58
10.	L997F	ARMS–PCR	58
11.	Δ 1507	ARMS–PCR	62
12.	Y122X	ARMS–PCR	56
13.	1078delT	ARMS–PCR	58
14.	1898+1G $\rightarrow$ A	ARMS–PCR	60

percentage of predicted values and FEV1 and FVC higher than 80 % and higher than 0.7 ratio of FEV1/FVC were defined as normal.

Screening for *CFTR* Gene Mutation

All cases and controls were screened for 24 *CFTR* gene mutations using the polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method. Venous blood samples were collected from the participants into an EDTA vial and DNA extraction was done using a standard phenol–chloroform method.

Twenty four mutations were screened for all the 500 subjects enrolled in the study. Genotyping of the mutations was done by ARMS–PCR and PCR–RFLP method.

Genotyping of R553X [22], G85E and R1162X [23], N1303K, 621+1G>T [24], 1717-1G $\rightarrow$ A [25], 3849+10 kb C>T [26], R75Q [27] mutations were done as reported in the given literature. Primers have been designed by PRIMER 3 Software for those mutations of which reported primers were not available in the literature. Tables 1 and 2 summarize the details of primer set used in the PCR and genotyping method and annealing temperature for the designed primers respectively.

Statistical Analysis

Data was collected on predesigned questionnaire. It was doubled entered in MS Excel, matched electronically and all discrepancies verified by checking with the hard data. Data was analyzed using SPSS software (Version 11.5, Chicago, IL). Univariate analysis was done to study the frequency distribution of the variables. Continuous variables were expressed as mean $\pm$ SD. Chi square and Students *t* test were performed to test the association between categorical and continuous variables.

Results

Two hundred and fifty asthma cases and age–sex matched controls were screened for 24 *CFTR* gene mutations in the study (Fig. 1). Of which 166 (66.4 %) were boys and 84 (33.6 %) girls. Table 3 illustrates the demographical and clinical profile of the subjects. Various factors were assessed and found period of exclusive breast feed for <6 months (OR = 1.7, 95 % CI = 1.182–2.416, *p* value = 0.005) and caesarean mode of delivery (OR = 2.0, 95 % CI = 1.272–3.245, *p* value = 0.003) significantly associated with asthma risk.

Abbreviation-* mutations in intronic region

Fig. 1 Positions of mutations within the *CFTR* gene

Table 3 Demographic profile of bronchial asthma cases and controls

Characteristics	Case (<i>n</i> = 250)	Control (<i>n</i> = 250)	<i>p</i> value
Age in months (mean ± SD)	78.22 ± 43.28	77.12 ± 41.02	0.582
Weight in kg (mean ± SD)	18.00 ± 7.42	17.84 ± 9.98	0.839
Height in cm (mean ± SD)	111.01 ± 21.12	111.53 ± 18.49	0.771
BMI in kg/m ² (mean ± SD)	14.16 ± 2.29	14.01 ± 7.39	0.763
Religion			
Muslim	50 (20.0)	45 (18.0)	0.648
Hindu	200 (80.0)	205 (82.0)	
Consanguinity	37 (14.8)	30 (12.0)	0.431
Preterm birth	24 (9.6)	14 (5.6)	0.128
Family type			
Nuclear	141 (56.4)	137 (54.8)	0.787
Joint	109 (43.6)	113 (45.2)	
Socio-economic status			
Lower class	70 (28.0)	65 (26.0)	0.687
Upper/middle class	180 (72.0)	185 (74.0)	
Animals in house	94 (37.6)	75 (30.0)	0.089
Curtains in house	246 (98.4)	240 (96.0)	0.175
Carpets in house	100 (40.0)	80 (32.0)	0.077
Exclusive breast feed			
<6 months	124 (49.6)	92 (36.8)	0.005
≥6 months	126 (50.4)	158 (63.2)	
Mode of delivery			
Caesarean	59 (23.6)	33 (13.2)	0.003
Vaginal	191 (76.4)	217 (86.8)	
Indoor smoking by smokers	90 (36.0)	62 (24.8)	0.009

Statistically significant values are denoted in bold

Association of *CFTR* Gene Mutations with Asthma as Compared to Controls

Among 24 mutations, heterozygous allele was found only in R553X mutation in 1.6 % (*n* = 4) among asthmatic cases and 0.4 % (*n* = 2) among controls. No significant

association was found (OR = 1.339, CI = 0.755–2.374, *p* value = 0.685). Furthermore, all wild type homozygous alleles were observed in remaining 23 *CFTR* gene mutations among asthmatic cases and controls. No heterozygous allele and mutant homozygous alleles were found in rest of the 23 mutations screened in the study.

Association of R553X Mutation with Severity of Asthma

Disease severity among asthma cases was categorized as per GINA guidelines. Of which 83 (33.2 %) belonged to mild intermittent, 96 (38.4 %) mild persistent, 52 (20.8 %) moderate persistent, and 19 (7.6 %) severe persistent category. Among 4 mutant individuals for R553X, 1 was mild intermittent, 1 was mild persistent and 2 were moderate persistent and none of the heterozygous individual belongs to severe persistent category. After comparing genotype frequencies between asthma severity subgroups and controls, no significant difference was observed between individuals mutant for R553X mutation in asthmatic cases as compared with controls. Details were summarized in Table 4.

Clinical symptoms (breathing difficulty, cough, wheeze, chest tightness, and disturbed sleep) and level of severity of asthma was observed among the cases with the R553X variants (*n* = 4) and those with wild type genotype (*n* = 246). Table 5 provided the details of clinical features in the heterozygous individuals for R553X mutations. No significant difference was found between them.

Association of R553X Mutation with Sweat Chloride Levels

Sweat chloride level was observed significantly higher among cases (*n* = 216, 22.39 ± 8.45 mmol/L) as compared with controls (*n* = 112, 19.55 ± 7.04 mmol/L) (*p* value = 0.048). When the individuals mutant for R553X mutations (*n* = 4, 27.75 ± 6.70 mmol/L) were compared with wild type individuals (*n* = 212, 22.24 ± 9.56 mmol/L), no significant difference in the sweat chloride levels was obtained (*p* value = 0.199).

Table 4 Association of R553X mutation with severity of bronchial asthma

Genotype	Control	Mild intermittent (<i>n</i> = 83)		Mild persistent (<i>n</i> = 96)		Moderate persistent (<i>n</i> = 52)		Severe persistent (<i>n</i> = 19)	
	<i>n</i> (%)	<i>n</i> (%)	OR (95 % CI); <i>p</i> value	<i>n</i> (%)	OR (95 % CI); <i>p</i> value	<i>n</i> (%)	OR (95 % CI); <i>p</i> value	<i>n</i> (%)	OR (95 % CI); <i>p</i> value
R553X Mutant	2 (0.8)	1 (1.2)	1.5 (0.131–6.902); 1.000	1 (1.04)	1.3 (0.113–14.571); 1.000	2 (3.84)	4.9 (0.681–6.062); 0.138	NC	NC
Wild Type	248 (99.2)	82 (98.8)		95 (98.95)		50 (96.15)			

OR odds ratio, CI confidence interval, NC not calculated

Table 5 Demographic and clinical profile of cases with R553X heterozygous allele

Gender	Age in months	Age of onset of disease in months	Consanguinity	Wheeze	Breathing difficulty	Cough	Disturbed sleep	Wheezing apart from cold	Previous episode of asthma	Previous use of bronchodilator	Family history of asthma	Severity of disease
P1 F	96	12	+	+	+	-	-	-	+	+	-	Intermittent
P2 M	120	36	-	+	+	+	+	-	+	+	+	Persistent
P3 F	92	05	-	+	+	+	-	+	+	+	+	Persistent
P4 F	124	18	-	+	+	+	-	-	+	+	-	Persistent

P patient, M male, F female, + present, - not present

Table 6 Percentage predicted FEV1, FVC and FEV1/FVC ratio in R553X mutation carrier and non-carriers

	Carriers (n = 4)	Non-carriers (n = 246)	p value
FEV1 % (mean ± SD)	68.49 ± 13.85	78.88 ± 11.67	0.092
FVC % (mean ± SD)	39.06 ± 16.60	52.51 ± 11.34	0.028
FEV1/FVC (mean ± SD)	0.55 ± 0.12	0.65 ± 0.09	0.041

Statistically significant values are denoted in bold

FEV1 forced expiratory volume on one second, FVC forced volume capacity

Association R553X Mutation with Spirometric Values

Spirometry was performed in 123 cases in children >6 years of age. Table 6 describes the lung function measurement details among carriers and non carriers. Among cases, when the percent predicted FVC and FVC/FEV1 ratio were compared between the individuals heterozygous for R553X with those of wild-type, value of FVC and FEV1/FVC ratio were significantly lower in heterozygous individuals compared to wild type homozygous individuals (p value <0.05).

Discussion

This study was conducted in 250 cases and 250 controls, to assess the association of *CFTR* gene mutations with asthma and its severity. Genotyping was done for 24 *CFTR* gene mutations, of which only in R553X mutation we have found heterozygous allele in cases and controls and was not found statistically significantly associated with asthma.

R553X, nonsense mutation located at exon 11 on *CFTR* gene. The R553X mutation substitutes an arginine codon for a stop codon, which would be expected to result in a severely truncated protein [28]. Among cases mutant allele frequency of R553X mutation was 1.4 %, whereas in control mutant allele frequency was 0.8 % respectively. This mutation was reported in cystic fibrosis cases like, from Chandigarh identified R553X mutation in one cystic fibrosis patient [29]. Worldwide frequency of this mutation was reported as 1.8 % in France, 2.9 % in Turkey 0.6 % in Bulgaria, 0.9 % in USA and 0.5 % in Mexico respectively in CF cases [20]; however its frequency has not been reported in asthmatic cases yet (Table 7).

In the present study we have not found any heterozygous or mutant homozygous allele in remaining *CFTR* gene mutations. Our results were similar as French [30], Italian [31], Singaporean Chinese [19] and Norwegian [32]

Table 7 Frequency of R553X mutation

Countries	R553X mutation (%)	Disease
Italy	1.03	Cystic fibrosis
European	0.26	
France	1.8	
Turkey	2.9	
Bulgaria	0.6	
USA	0.9	
Mexico	0.5	Asthma
Chandigarh	2	
Current study	1.6	

populations. In contrast, some other studies reported the positive association of F508 heterozygosity with an increased susceptibility to asthma [16, 33, 34] while protective association has also been reported [18]. In a study by Awasthi et al., G551D mutation was found associated with asthma which was the third most common mutation worldwide but no significant association of DF508 was observed among the asthma group [21].

Lung function test was done to assess the percent predicted FEV1 and FVC. A statistically significantly lower frequency of percentage predicted FEV1 and FVC were observed among carriers as compared to non-carriers in asthmatics. Like-wise other studies done in different populations who have shown that *CFTR* hetero-zygosity may be associated with decreased pulmonary function in people with asthma [35–37]. While other studies have observed a similar distribution of percent predicted FEV1 and FVC in the carriers and non-carriers [32, 38, 39].

We found a significant association of period of exclusive breast feed for <6 months with increased asthma risk in our study. Similarly it was reported previously also that prevalence of asthma was low in children with exclusive breast feed compared to infants with partial breast feeding and formula milk [40–42]. Unlike these results, higher prevalence of asthma was found among children who had been breastfeed [43] and also reported that breastfeeding does not protect children against atopy and asthma and may even increase the risk [44]. Likewise, we observed caesarean mode of delivery was found significantly associated with asthma risk, as reported earlier also [45, 46]. Unlike these studies, some studies have reported that mode of delivery is not associated with subsequent risk of developing childhood asthma or wheezing episodes [47, 48].

The strength of our study is that the sweat chloride test was performed to exclude cystic fibrosis cases misclassification arising due to some common symptoms of asthma and CF, i.e. wheeze and Bronchial Hyper Responsiveness. However, the drawback of our study is that the spirometry test was not performed in the control group which could

give a better explanation regarding *CFTR* heterozygosity and pulmonary abnormalities in general population.

In conclusion we found that R553X mutation of *CFTR* gene was not associated with asthma however, lower values of percent predicted FVC and FEV1/FVC ratio was found in carriers as compared to non-carriers which shows that carriers may be at risk for poor pulmonary function.

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