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Allergic Chronic Rhinosinusitis: A Look at NAT2A Gene Polymorphisms

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Running Title: Association of NAT2A gene polymorphism with allergic CRS

Abstract: ***Recent studies exploring the genetic factors associated with allergic chronic rhinosinusitis are of great importance. However, the role of NAT2A polymorphism in CRS remains relatively understudied. To address this gap in knowledge, we conducted a research study examining the association between NAT2A polymorphism and allergic chronic rhinosinusitis. Our study aimed to investigate the prevalence of NAT2A polymorphisms in patients with allergic chronic rhinosinusitis (n=124) and Controls (n=175) to determine whether these genetic variations are linked to the development and severity of the condition. We recruited a sample of patients diagnosed with allergic chronic rhinosinusitis and genotyped them for NAT2A polymorphisms. Additionally, we collected data on their symptoms, comorbidities and disease severity. Our findings revealed a significant association between NAT2A polymorphism and allergic chronic rhinosinusitis. GA and AA genotype of Taq I 590 G>A (rs 1799930 Arg197Gln) indicated a risk of 4 times to 3 times in allergic CRS subjects. Further, the haplotype NAT2*6A NAT2*7A genotype, associated with slow acetylation was a significant risk of 2 folds for allergy. Specifically, we found that individuals with slow variants of the NAT2A gene were more likely to develop allergic chronic rhinosinusitis.***

Keywords: Allergic chronic rhinosinusitis, NAT2A polymorphism, genetic association, disease severity, slow acetylation variants

1. Introduction

Chronic Rhinosinusitis (CRS) is a complex inflammatory condition affecting the nasal and paranasal sinuses, with a global impact on millions of individuals. Various factors, including anatomical, microbial, genetic, and allergic factors, are believed to contribute to the development of CRS. Oxidative stress induced by air pollutants and allergens can trigger allergic symptoms and inflammation in the nasal and sinus tissues (Gilmour et al., 2006, Huang et al., 2016). To counteract oxidative stress, the phase I and phase II antioxidant enzymes plays a role in metabolizing xenobiotics involving drug and food component oxidation, hydrolysis, and reduction. One such phase I enzyme is N - acetyl transferase 2 (NAT2A), is located on chromosome 8p 21.3 - 23.1 and consists of a single exon with an open reading frame of 870bp, encoding a protein with 290 amino acids (Valko et al., 2007). NAT2A gene expression is predominantly observed in the lungs, colon, breast, prostate, liver, and gut ((Windmill *et al.,* 2000). It catalyzes the transfer of an acetyl group from acetyl CoA to the nitrogen of substrate. Polymorphisms at the *NAT2* gene locus result in impaired enzyme activity (Borlak and Reamon - Buettner, 2006). There are three *NAT2* phenotypes: The fast acetylation phenotype which consists of two copies of wild type allele (WT), an intermediate phenotype with one wild type allele and a mutant allele, and a slow acetylator phenotype comprising two mutated alleles. Individuals homozygous or heterozygous for the *NAT2* WT allele are classified as rapid acetylators whereas those homozygous for any of the remaining (mutant) alleles are classified as slow acetylators (Seow *et al.,* 1999). Mutated alleles of NAT2 typically encode proteins with reduced enzymatic activity and have been associated with various conditions such as prostate cancer, gastrointestinal tract cancer, urinary bladder cancer, systemic lupus erythematosus (SLE), endometriosis, Parkinson's disease, and Alzheimer's disease. Polymorphisms occurring at the NAT2 gene locus can lead to impaired enzyme activity. Based on seven different mutations in the NAT2 gene (191G/A, 282C/T, 341T/C, 481C/T, 590G/A, 803A/G, and 857G/A), individuals can be categorized as either rapid or slow acetylators. Rapid acetylators refer to individuals homozygous or heterozygous for the NAT2∗4 allele, who have a higher rate of metabolic activation of aryl aromatic amines. In contrast, slow acetylators include individuals homozygous for NAT2*5A, *5B, *5C, *6A, and *7B alleles, characterized by a lower rate of metabolic activation of these amines (Borlak and Buettner, 2006; Naazneen et al., 2013). The proportion and frequency of fast and slow acetylation phenotypes exhibit significant variation depending on ethnicity and geographic origin. For instance, populations in Europe and North America display a prevalence of 40 - 70% slow acetylators, while Asian populations such as Turkish, Japanese, Chinese, and Koreans have a lower proportion of slow acetylators, ranging from 10 to 30% (Meyer and Zanger, 1997, Garte *et al.,* 2001, Nacak et al., 2002). Studies conducted on Indian populations have also reported significant differences in the frequency of mutant alleles of xenobiotic metabolizing enzyme (XME) genes, including NAT2A (Umamaheswaran

et al., 2014). While there is evidence suggesting an association between NAT2 gene variants and respiratory diseases and allergies, further investigations involving diverse populations are warranted. Further, limited information is available regarding the allele and genotypic frequencies of NAT2A in chronic rhinosinusitis. Therefore, the present study aimed to examine the role of NAT2A gene variants in the development of allergic CRS.

2. Methods

In this case - control study, we conducted molecular evaluation on 124 adult subjects with allergic chronic rhinosinusitis (aCRS) and 150 healthy controls, following the guidelines of the European Position Paper on Rhinosinusitis and Nasal Polyps (EP3OS) (Fokkens et al., 2012). All the study participants were diagnosed and confirmed by ENT specialists at MAA ENT Hospitals in Hyderabad. Subjects with a high level of total IgE (>180 IU, measured by ELISA) and exhibiting allergic symptoms were classified as allergic CRS subjects. Controls were individuals with normal total IgE levels (<180 IU) and no history of allergy, asthma, or CRS. Subjects with other syndromes such as cystic fibrosis, immunodeficiency diseases, immune - compromised conditions, nosocomial infections, and allergic fungal sinusitis were excluded from the study. Informed written consent was obtained from all participants, and the study was approved by the institutional ethics committee.

For genotyping, approximately 2 ml of whole blood was used for DNA extraction using the salting - out method (Laheri et al., 1991). The PCR was carried out in a 25µl reaction mixture containing 1U of thermostable Taq DNA polymerase. We used nested PCR - RFLP based on the protocol described by Cascorbi et al., 1995 to determine the genotypes of NAT2 BamHI 857 G>A (rs1799931 Gly286Gln), KpnI 481 C>T (rs1799929), FokI 282 C>T (rs1041983), TaqI 590 G>A (rs1799930 Arg197Gln), DdeI 803 A>G (rs1801280 Ly268Arg). The PCR products of 1211 bp were generated using specific primers, and the resulting mixture underwent denaturation, annealing, and extension steps. The reaction mixture was subjected to initial denaturation at 94°C for 5 min, followed by 35 cycles (94°C, 1 min), annealing (58.5°C, 1 min) and extension (72°C, 1 min). The final extension was done at 72°C for 10 min. Following PCR, 7 µl of PCR products were digested with four separate enzymes including Kpn1 for NAT2*5 allele, at 37°C for 2 hrs; Taq1 for NAT2*6 allele, at 56°C for 4 hrs; BamH1 for NAT2*7 allele at 37°C for 2 hrs; DdeI for NAT2*14 and Fok1 for NAT2*13 allele at 37°C for 2 hrs. Digested product was run on 2% agarose gel for NAT2*5, NAT2*7, NAT2*14 alleles and 3% agarose gels for NAT2*6 allele. The variant and non - variant NAT2 alleles were recorded and rapid or slow acetylator phenotype assignments were deduced on the basis of NAT2 genotype (Srivastava et al., 2007). Genotypes possessing two variant alleles (NAT2*5, NAT2*6, NAT2*7, or NAT2*14) were assigned as slow acetylator phenotype whereas others were assigned as rapid acetylator phenotype. The PCR conditions, specific forward primer and reverse primer used and RFLP conditions are given in Table S1 - S3.

3. Statistical analysis

Differences in genotype prevalence and association between case and control groups were assessed by binary logistic regression model. Odds ratios (OR) and its 95% confidence interval (CI) were obtained by summarizing data over two habit strata (tobacco users/ non - users). The genotype and allele frequencies were compared between patients and controls using SPSS STATISTICS 21 software (SPSS Inc., Chicago, IL, USA). Genotype frequency distributions were tested for agreement with the Hardy - Weinberg equilibrium (HWE) performing a Pearson's χ^2 - test using electronic calculator [http: //www.genes. org. uk/software/hardy - weinberg. html]. Allelic and genotypic frequencies of all the above gene polymorphisms were quantified by the odds ratio (95% CI) and the Statistical significance of the differences in frequency of alleles, genotypes and genotypic models of inheritance; dominant, recessive, co - dominant, over - dominant and log additive models., were carried out using logistic regression analysis for all the SNPs individually. $P < 0.05$ was considered statistically significant. The power of the study was calculated for individual SNPs using G*Power software (version 3.1, Universitat Dusseldorf, Germany). Genotype frequencies for all the SNPs under study were in HWE in cases and controls.

4. Results

A total of 287 subjects participated in this study, including 124 individuals with allergic chronic rhinosinusitis (CRS) (64 males and 60 females) and 175 healthy controls (94 males and 81 females). The mean age of the allergic CRS subjects was 36.4 ± 14.10 years (ranging from 18 to 68 years), while the mean age of the healthy controls was 48.22 ± 11.26 years (ranging from 18 to 80 years). The mean age of onset for allergic CRS subjects was reported as 24 ± 9.42 years. The genotype frequency distributions were tested for agreement with the Hardy - Weinberg equilibrium (HWE). The study findings demonstrated that the GA and AA genotype of Taq I 590 G>A (rs 1799930 Arg197Gln) indicated a risk of 4 times to 3 times in allergic CRS subjects. Futher, the haplotype NAT2*6A and NAT2*7A genotype, associated with slow acetylation, was a significant risk of 2 folds for allergy. Among the other analyzed SNPs, N Acetyl Transferase 2A 857 G>A, Fok I 282 C>T, Kpn I 481 C>T, Taq I 590 G>A showed no association with allergic CRS. The results of the study indicate that the NAT2*6A and NAT2*7* genotypes, associated with slow acetylation, are important risk factors for allergic Rhinosinusitis in Table 1 while Table 2 represents the haplotype distribution, Figure 1 and the additional supplementary tables S1 to S3 provide further details on the nested PCR RFLP.

Table S1: Oligonucleotide primer pairs used for Nested PCR amplification of N acetyl Transferase 2A (NAT2A) gene polymorphism

Primer	Primer Sequence 5' - 3'
P100	5' - GTCACACGAGGAAATCAAATGC - 3'
P56	5' - GTTTTCTAGCATGAATCACTCTGC - 3'
P87	5' - CCTGGACCAAATCAGGAGAG - 3'
P90	5' - ACACAAGGGTTTATTTTGTTCC - 3'
P341	5' - ACCCAGCATCGACAATGTAATTCCTGCCCTCA - 3'

Table S2: PCR conditions for identification of polymorphism in N acetyl Transferase 2A (NAT2A) gene

Mutation Position	Primers used	Initial Denaturation	Denaturation	Annealing	Elongation	Final Elongation	No. of Cycles
rs1799929, rs1799931	P100/P56	94°C for 10 min	94°C for 30 sec	56 °C for 1min	72°C for 2 min	72°C for 5 min	35
rs1799930, rs1208	P87/P90	94°C for 10 min	94°C for 30 sec	57°C for 30sec	72°C for 30sec	72°C for 5 min	14
rs1041983, rs1801280	P100/341	94°C for 10 min	94°C for 30 sec	60°C for 30sec	72°C for 30sec	72°C for 5 min	14

Table S3: Restriction endonucleases used for determination of six mutations of the N acetyl Transferase 2A (NAT2A) gene and characteristic patterns of respective DNA fragments

rs Identifier	Position and Nucleotide Change	Restriction Endonuclease (5U)	Primers	Temp & Incubation time	Agarose Gel (%)	Fragment pattern (bp)
rs1041983	282 C>T	FokI	P100/P341	65°C for 2hrs	2	CC: 337, 105; TT: 442
rs1801280	341T>C	DdeI	P100/P341	37°C overnight	4	TT: 220, 163, 59; CC: 188, 163, 59, 32
rs1799929	481C>T	KpnI	P100/P56	37°C overnight	2	CC: 662, 549; TT: 1211
rs1799930	590 G>A	TaqI	P87/P90	65°C for 2hrs	3	GG: 170, 142, 109; AA: 279, 142
rs1208	803 A>G	DdeI	P87/P90	37°C overnight	4	AA: 297, 124; GG: 297, 97, 27
rs1799931	857 G>A	BamHI	P100/P56	37°C overnight	2	GG: 925 286; AA: 1211

Table 1: Frequencies of NAT2A allelic and genotype and their associated risk in the study subjects

Inheritance models	Genotypes	Controls (%)	aCRS	Odds Ratio (95% CI)	P - value
BamHI 857 G>A (rs1799931 Gly286Gln)					
Co - dominant	G/G	126 (72%)	80 (65%)	1	0.18
	G/A	38 (21.7%)	28 (22.8%)	1.16 (0.66 - 2.04)	
	A/A	11 (6.3%)	15 (12.2%)	2.15 (0.94 - 4.91)	
Dominant	G/G vs G/A+ A/A	49 (28%)	43 (35%)	1.38 (0.84 - 2.27)	0.02
Recessive	G/G+G/A vs A/A	11 (6.3%)	15 (12.2%)	2.07 (0.92 - 4.68)	0.078
Over - dominant	G/G+A/A vs G/A	38 (21.7%)	28 (22.8%)	1.06 (0.61 - 1.85)	0.83
Allele Frequencies	G	290 (83.0)	188 (75.0)		
	A	60 (17.0)	58 (25.0)		0.002
Kpn I 481 C>T (rs1799929)					
Co - dominant	C/C	64 (36.6%)	53 (43.1%)	1	0.33
	C/T	65 (37.1%)	46 (37.4%)	0.85 (0.51 - 1.44)	
	T/T	46 (26.3%)	24 (19.5%)	0.63 (0.34 - 1.16)	
Dominant	C/C vs C/T+T/T	111 (63.4%)	70 (56.9%)	0.76 (0.48 - 1.22)	0.26
Recessive	C/C+C/T vs T/T	46 (26.3%)	24 (19.5%)	0.68 (0.39 - 1.19)	0.17
Over - dominant	C/C+T/T vs C/T	65 (37.1%)	46 (37.4%)	1.01 (0.63 - 1.63)	0.96
Allele Frequencies	C	193 (54.6)	152 (61.8)		
	T	157 (45.4)	94 (38.2)		
Fok I 282 C>T (rs1041983)					
Co - dominant	C/C	48 (27.4%)	30 (24.4%)	1	0.76
	C/T	88 (50.3%)	67 (54.5%)	1.22 (0.70 - 2.12)	
	T/T	39 (22.3%)	26 (21.1%)	1.07 (0.54 - 2.09)	
Dominant	C/C vs C/T+T/T	127 (72.6%)	93 (75.6%)	1.17 (0.69 - 1.99)	0.56
Recessive	C/C+C/T vs T/T	39 (22.3%)	26 (21.1%)	0.93 (0.53 - 1.64)	0.819
Over - dominant	C/C+T/T vs C/T	88 (50.3%)	67 (54.5%)	1.18 (0.74 - 1.88)	0.48
Allele Frequencies	C	184 (52.6)	127 (51.4)		
	T	166 (47.4)	119 (48.6)		0.702
Taq I 590 G>A (rs 1799930 Arg197Gln)					
Co - dominant	G/G	81 (46.3%)	20 (16.3%)	1	
	G/A	78 (44.6%)	90 (73.2%)	4.67 (2.63 - 8.31)	<0.001
	A/A	16 (9.1%)	13 (10.6%)	3.29 (1.36 - 7.94)	
Dominant	G/G vs G/A+A/A	94 (53.7%)	103 (83.7%)	4.44 (2.53 - 7.80)	<0.001
Recessive	G/G+G/A vs A/A	16 (9.1%)	13 (10.6%)	1.17 (0.54 - 2.54)	0.688
Over - dominant	G/G+A/A vs G/A	78 (44.6%)	90 (73.2%)	3.39 (2.06 - 5.58)	<0.001
Allele Frequencies	G	240 (68.5)	130 (53.0)		
	A	110 (31.5)	116 (47.0)		<0.001

DdeI 803 A>G (rs1801280 Ly268Arg)					
Co - dominant	A/A	76 (43.4%)	66 (53.7%)	1	
	A/G	84 (48%)	45 (36.6%)	0.62 (0.38 - 1.01)	0.14
	G/G	15 (8.6%)	12 (9.8%)	0.92 (0.40 - 2.11)	
Dominant	A/A vs A/G+G/G	198 (56.7)	115 (45.9)	0.71 (0.49 - 1.03)	0.082
Recessive	A/A+A/G vs G/G	31 (8.9)	24 (9.5)	1.22 (0.65 - 2.31)	0.73
Over - dominant	A/A+G/G vs A/G	167 (47.8)	91 (36.4)	0.66 (0.45 - 0.96) *	0.05
Allele Frequencies	A	471 (67.2)	361 (72.3)		
	G	229 (32.8)	139 (27.7)		
a. OR - Odds ratio; CI - Confidence interval, c. *p - value <0.05; **p - value <0.01; ***p - value <0.001					

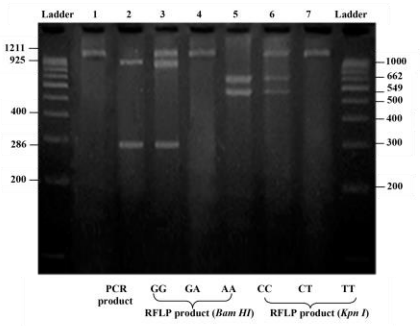
Table 2: Frequencies of NAT2A haplotypes and their associated risk in the study subjects

Haplotypes	Control	aCRS	OR (95% CI)	p - value
Fast acetylator				
NAT2 *4	57 (32.6)	22 (20.0)	1.00 (Reference)	
Slow acetylator				
NAT2 *6	28 (15.7)	39 (35.0)	2.40 (1.54 - 3.73) ***	0.021
NAT2 *7	20 (11.5)	15 (13.2)	1.87 (1.00 - 3.49) *	0.043
NAT2 *12	21 (11.9)	3 (2.7)	0.38 (0.15 - 0.97)	0.621
NAT2 *13	4 (2.3)	3 (2.7)	2.00 (0.61 - 6.56)	0.121
NAT2 *5	42 (24)	22 (20)	0.910 (0.39 - 10.32)	0.521

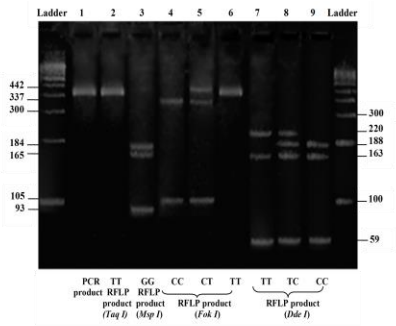
* Level of significance with p value less than 0.05,
* * *Level of significance with p value less than 0.05

5. Discussion

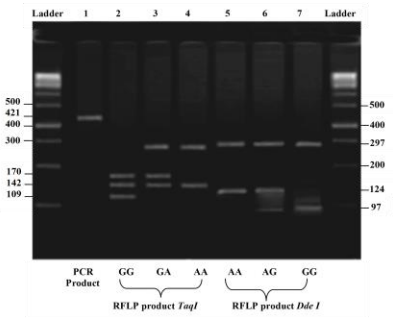
Allergies and chronic rhinosinusitis (CRS) are becoming more common worldwide, and the complex interactions between hereditary and environmental factors greatly influence the allergic phenotypes. The inter individual variations in reactive metabolite production of atopy and CRS may be significantly influenced by the enzyme family known as arylamine N - acetyltransferases (NATs), which is essential for the biotransformation and detoxification of xenobiotics (Elena Garcia et al., 2008).



Bam HI and Kpn I digest of 1211 bp fragment containing polymorphism at G481A and C857T of NAT2



Taq I, Msp I, Fok I and Dde I digest of 442bp fragment containing polymorphism at G590A and A803G of NAT2



Taq I and Dde I digest of 421 bp fragment containing polymorphism at G590A and A803G of NAT2

Supplementary Figure 1: Gel Picture of NAT2A Polymorphism

The function of NAT2A enzymes can be affected by single nucleotide polymorphisms (SNPs). Further, significant intraethnic variability for NAT2A SNPs was detected for the SNPs G191A, T341C, C481T and G590A among African and Oriental individuals while no significant variations were found with respect to C282T, A806G, or G857A SNPs. Interethnic variability was observed for all SNPs except C282T (Tamer et al., 2006. Zielińska et al., 1997).

Populations from Jordan, Europe, and Africa exhibit a higher frequency of slow acetylator phenotypes, whereas groups from China, Japan, and the Americas tend to exhibit a higher prevalence of rapid acetylator phenotype. The slow allele of NAT2A is present in 5 - 25% of East Asians, 40 - 20% of Caucasians and up to 90% of Arab populations. A research conducted in on NAT2A genotyping of Dravidian ethnic communities in South India showed that 74% of them were slow acetylators, with allele 6A being the most common which is consistent with the frequency seen in Caucasians. In a study conducted by Tilak et al., 2013 the slow acetylator haplotypes was 65% while Srivastava et al.2005 showed a rapid acetylator genotypes to be more commonly seen in 64.6% of subjects. A novel deletion at the 859 site was also linked to a homozygous mutation at the 481 site. The polymorphism of NAT2A at T111C, G191A, A434C, and C759T was reported as non - polymorphic in the North Indian population, with a robust linkage disequilibrium among the T341C, C481T, and A803G polymorphisms (Batra et al, 2006).

Schnuch et al. suggested that acetylation may enhance contact sensitization, influencing the interplay between

NAT2 polymorphism and allergy/atopy. Previous studies indicate a link between slow acetylation and a predisposition to atopic diseases, while others note a higher proportion of rapid acetylators in patients with contact allergic dermatitis (Tamer et al., 2006. Zielińska et al., 1997). Gawrońska et al., 1999 and Gawronska et al.2001 study showed that Homogygous slow acetylators in the Polish population had a five - fold increased risk of developing atopic disorders in both adult and pediatric population and Slow acetylators had an almost three - fold higher chance of developing an immunoglobulin E - mediated food allergy than did healthy patients (Tamer et al., 2006), The NAT2*5A acetylator phenotype susceptibility to bronchial asthma was more than two times higher.

Luszawska - Kutrzeba (1999) in Polish population found significant risk factor for bronchial asthma in the white Polish group was slow acetylation of the NAT2* genotype. Zielińska et al., 1997; Nacak and associates, 2002 noted that NAT2A polymorphism showed modest atopic asthma in the Turkish population. Further, the risk of developing immunoglobulin E - mediated food allergy was nearly three times higher in slow acetylators than in healthy subjects. NAT2 5A and NAT2 6A, were also associated with increased risks for bronchial asthma. In the North Indian population, genetic variants C481T and C282T of the NAT2 gene were reported to affect asthma and modulate asthma - related traits, including serum IgE and blood eosinophil counts (Batra, 2006). Consistent with the above studies the present study also Taq I 590 G>A finds NAT2 influence the development allergic CRS by 4 times when compared to normal controls. However, Nacak et al., 2002 suggested minor role of NAT2 polymorphism in atopic asthma in the Turkish population and indicated the nuanced influence of various factors, including specific allergens in different regions and the underlying etiology of the allergy, on the relationship between NAT2 polymorphism and atopy. A study by Yilmaz et al. [21], indicated that in adults of Turkey the allergens that trigger allergy reactions differs significantly in children from other European countries.

6. Conclusion

In conclusion, NAT2 polymorphism appears to influence susceptibility to allergic CRS, but the nuanced relationship between acetylation rates and the development of allergic CRS requires further exploration. This is the first study to report the association of NAT2A gene variants with allergic CRS in Indian population.

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