ORIGINAL ARTICLE

RISK LOCI FOR CHRONIC OBSTRUCTIVE DISEASE RESIDE ON CHROMOSOME 14: A CASE-CONTROL STUDY ON THE PAKISTANI POPULATION

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Background: Chronic Obstructive Pulmonary Disease (COPD), the third leading cause of death worldwide, is characterized by airflow limitation that can be due to abnormalities in the airway and/or alveoli. Genetic diagnosis at an early stage can be a key factor in the provision of accurate and timely treatment. Single Nucleotide polymorphisms (SNPs) are an important tool to study genetic association/ predisposition of the disease and have great potential to be diagnostic markers for early diagnosis of disease. **Methods:** This case-control COPD association study was designed for the five SNPs residing on potential candidate genes (*SERPINA1*, *SERPINA3*, *RIN3*), to check whether these genes are involved in the genetic predisposition for COPD in the Pakistani population or not. The SNAPshot method was used to find out the risk alleles and haplotypes using ABI Genetic analyzer 3130. GeneMapper, Haploview and PLINK 1.9 software were used for analyzing the genotypes and haplotypes taking smoking exposure and gender as covariates. **Results:** Two of the SNPs, rs4934 and rs17473 were found to be independently and significantly associated with COPD in our studied population whereas haplotype H1 for two SNPs, rs754388 and rs17473 (that are in high linkage disequilibrium), was found to be a significant risk factor for developing COPD symptoms. **Conclusion:** SNP variants of *SERPINA1* and *SERPINA3* are significantly and independently associated with COPD in the local population of Pakistan.

Keywords: COPD; *SERPINA*; Single Nucleotide Polymorphism; Association study

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INTRODUCTION

Among non-communicable diseases, Chronic Obstructive Pulmonary Disease (COPD) is the third leading cause of death worldwide**.** According to World Health Organization COPD is defined as "a common, preventable and treatable disease that is characterized by persistent respiratory symptoms and airflow limitation owing to airway and/or alveolar abnormalities usually caused by significant exposure to noxious particles or gases". According to an estimate in 2019, GOLD-COPD prevalence in ageing people between 30–80 was 10.3% in high income countries and was even higher in low middle income countries. Overall, the prevalence was observed to be 14.1% in men and 6.5% in women.[1] More than 384 million of cases exist globally, with around three million deaths due to COPD.[2] The mortality is expected to rise upto 5.4 million per annum in the next 40 years.[3] An epidemiological study done on East-Asian countries showed a 2.1% of COPD prevalence in Pakistani elderly people.[4] Recently, few area and population-restricted studies have even shown a higher prevalence of 6.7%, 11.31% and 19.4% in the ageing population of Pakistan.[5–7] Pathologically COPD is divided into two major types, either patients with alveolar wall destruction (emphysema) or with inflammation of the bronchi (Chronic Bronchitis) and some have both conditions. Diagnosis of COPD patients is commonly done by pulmonary function test/Spirometry with FEV1/FVC ratio of <70% and in some cases with computerized tomography scans, if available.[8]

The only known genetic factor responsible for the disease is *SERPINA1*, located on 14q23, which encodes the AAT1 protein, also known as an alpha1-protease inhibitor (A1PI) or alpha1 anti-protease (A1AP). Under normal conditions, this enzyme is released by white blood cells to fight infection, but it can also attack normal lung tissues if not controlled by alpha-1 antitrypsin. Malfunctioning of alpha-1 antitrypsin enzyme damages alveoli and causes lung disease.[9–13] Abnormal alpha-1 antitrypsin accumulation in the liver can also damage this organ.[14] Alpha-1 antitrypsin deficiency severity is enhanced by many environmental factors like tobacco smoke, chemicals, and dust.[15] Diane *et al* confirmed that low levels of AAT1 are associated with

the early onset of COPD[16]; however, this deficiency has been reported in only 2–3% of the diagnosed cases.[17] It is the need of an hour to conduct studies for exploring other genetic factors that might be causing the symptoms.

More than 125 SNPs have been reported in various public databases making this gene highly pleomorphic.[18] A study reported an association between SNP rs17580 of this gene and α-1 Anti Trypsin level in serum.[19] Similarly, in another study variants associated with emphysema, a subtype of COPD, were found. Only rs28929474 is found to be strongly associated with lung function.[20–23]

RIN3 is a member of the RIN family of Ras interaction-interference protein which is a binding partner to the RAB5 small GTPase. It is a protein-coding gene residing on chromosome 14 and is 175,222 bases long. The function of this gene is endocytic and endocytosis activity by exchanging bound GDP and guanine nucleotide (GEF).[24] Previous studies reported an association of the *RIN3* gene with Paget's disease and Alzheimer's disease, but in a recent meta-analysis, done by Cho and colleagues in the general population of European ancestry from UK Bio bank, identified *RIN3* association with COPD at a significant level.[25] This study helped in COPD diagnosis definition and its severity determination in smokers by measurement of the effect of post-bronchodilator on FEV_1/FVC and FEV_1 values. A similar study on Non-Hispanic whites, with current and former smokers, has also shown associated SNPs in this genomic region.[26]

Our Southeast Asian region differs from other continents in its genetic makeup, ancestry, ethnicity, environmental conditions, poor socio-economic background, history of repeated childhood respiratory tract infections and biomass exposure. No elaborative studies on potential candidate genes, i.e., *SERPINA1*'s SNP/haplotype association with COPD, and its subtypes, have been conducted in any of the Pakistani populations till now to the best of our knowledge. Also, the linkage disequilibrium pattern for this specific region in our Pakistani population is not known. So, the primary objective of this case-control COPD association study was to find out risk alleles and genotypes of potential candidate genes, that may predispose our population to COPD.

MATERIAL AND METHODS

A total of two-hundred and fifty-four COPD patients were enrolled between the year 2018 to 2020 from different hospitals in Lahore after approval from the relevant Ethics Review Board (138/DFEMS). A total of two-hundred and twenty-seven, age and gender-matched, healthy controls were selected after screening from filter clinical units of these hospitals. Informed consent were obtained from all the individuals participating in the study.

COPD follow-up patients visiting outdoor clinic of Pulmonology department at Fatima Memorial Hospital were enrolled for this study. These patients were diagnosed by collaborating clinicians based on their clinical parameters including Chest Xrays, FEV values, number of exacerbations, medical history etc. Facts relevant to age, family history, cast, primary and secondary smoking exposure and other social and economic factors related to disease were recorded in a predesigned standardized questionnaire for each individual. Table 1 shows the demographic characteristics of enrolled patients. Patients with a history of hepatitis, tuberculosis and other infectious diseases were excluded from the study. Similarly, controls with a history of any contagious or respiratory disease were also excluded.

Standard protocol was used for taking blood samples of both the controls and the patients. The phenol-chloroform protocol was used for genomic DNA extraction from peripheral blood leukocytes. Quantification of extracted DNA was done by agarose gel electrophoresis.

Five potential risk-associated SNPs: rs754388, rs4934, rs17473, rs1800463 and rs1303, residing on chromosome 14 between 92649065 bp to 94619305 bp either within or among three selected genes *SERPINA1, SERPINA3 and RIN3*, were studied to evaluate their possible association with emphysema in Pakistani population (Table S1).

Targeted genomic regions, with selected SNPs, were amplified in a multiplex polymerase chain reaction. A list of primers and their concentrations used for optimizing the multiplex PCR is given in Supplementary Table S2. The concentration of reagents used for multiplex reaction along with the volume used is provided in supplementary data (Table-S4). Samples were denatured at 95 ºC for 5 minutes. Amplification was done by 30 cycles of 95 ºC for 45 seconds, 58 ºC for 45 seconds and extension at 72ºC for 90 seconds, followed by a final extension at 72 ºC for 10 minutes.

5µl of obtained product from the above reaction was purified with the help of 1U of Exonuclease I (Fermentas) and 1.66 U of Shrimp Alkaline Phosphatase (Fermentas) by incubation at 37ºC for one hour followed by heating at 80 °C for 15 minutes.

Single base extension primers for selected five SNPs were designed by using Batch Primer 3 Software (MA, USA). ABI Prism SNaPshot™ Multiplex Kit (Applied Biosystems, Foster City, CA, USA) was used to genotype these SNPs. A list of primers and their concentration used for single base extension (SBE) PCR are given in Supplementary Table-S3. For the SBE reaction, incubation was done for twenty-five cycles at 96ºC for 10 seconds, 58ºC for 5 seconds and 60ºC for 30 seconds after mixing all necessary reagents (recipe of mix in supplementary data). Final purification was done with 1 unit of Shrimp alkaline Phosphatase enzyme by

incubating at 37ºC for an hour followed by denaturation by heating at 75ºC for 15 minutes. For genotyping on ABI 3130xl, 1µl of SAP purified SNaPshot product along with 10µl of Hi-Di Formamide and 0.1µl of Gene-Scan 120 LIZ internal size standard (Applied Biosystems) were denatured at 95ºC for 5 minutes. ABI GeneMapper ID-X software (Applied Biosystems) was used to identify peaks for different genotypes of selected SNPs. PLINK v1.9 [12] was used to find the possible associations under additive, dominant and recessive models for each SNP marker. Individuals with a low genotyping call rate [i.e., < 90%] were excluded from the analysis. p-values for logistic regression were adjusted for gender and smoking exposure as covariates. Pair-wise Linkage Disequilibrium map was constructed with Haploview v4.1 (Figure-1) and the haplotype analysis was done for SNPs having higher linkage disequilibrium with each other (Table-4).

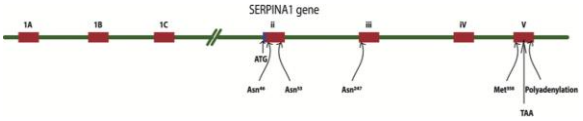

Figure-2: Genomic organization of *SERPINA1* gene:

SERPINA1 gene has 3 non-coding exons denoted as IA, IB, IC and 4 coding exons denoted as II, III, IV and V located on the q-arm of 14th chromosome. The critical reactive center loop is located in exon V of *SERPINA1* gene.

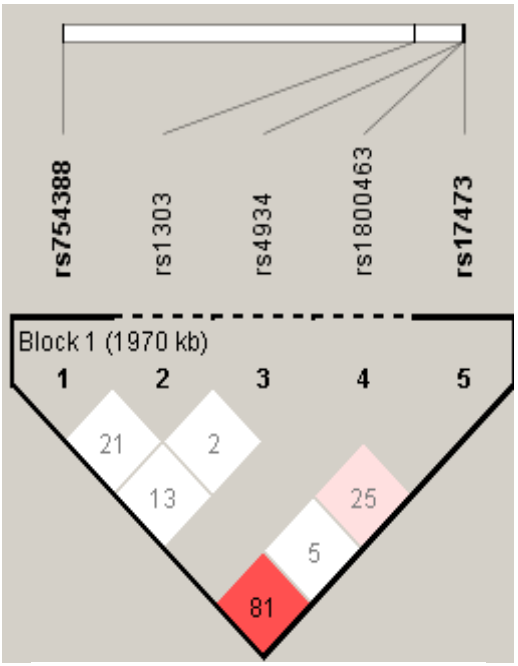

Figure-1: Linkage Disequilibrium block constructed by Haploview4.1

Table-1: Demographic Characteristics of the studied Population

Variable	Cases	Controls
No of Individuals	254	227
Age Range (Years)	22-75	20-70
Gender (%Male)	55.1%	55.3%
Smoking history	57.08%	14.1%
Positive Family History	36.6%	0%

Table-2: Association tests of SNPs under allelic, dominant and recessive models on PLINK v1.9

SNP	Reference allele	Second Allele	TEST	*CHISQ*	p-Value
rs754388	T	C	Allelic Dominant Recessive	0.8342 0.4569 0.443	0.361 0.499 **0.0505***
rs1303	C	T	Allelic Dominant Recessive	1.346 3.644 0.1155	0.246 0.056 0.734
rs4934	G	A	Allelic Dominant Recessive	9.176 0.593 13.06	**0.0024*** 0.440 **$3.0x10^{-4}$***
rs1800463	0	T	Allelic Dominant Recessive	NA NA NA	NA NA NA
rs17473	T	C	Allelic Dominant Recessive	7.374 4.173 12.7	**$6.0x10^{-3}$*** **0.041*** **$3.0x10^{-4}$***

CHISQ: Chi-Square value,* denotes *p*-value less than 0.05

Table-3: Logistic Regression analysis by PLINK1.9

SNP	A1	*p*-Value	OR	95%CI	Gender Adjusted OR	Gender Adjusted *p*-value	smoking Adjusted OR	smoking Adjusted *p*-value
rs754388	T	0.8238	0.955	[0.6365- 1.433]	0.868	0.445	0.866	0.546
rs1303	C	0.2165	1.155	[0.9191- 1.451]	1.089	0.402	1.125	0.368
rs4934	G	**0.04454***	1.233	[1.005- 1.514]	1.227	**0.019***	1.791	**$5.13x10^{-7}$***
rs1800463	0	NA	NA	NA	NA	NA	NA	NA
rs17473	T	**$1.286x10^{-4}$***	0.5265	[0.3791- 0.7312]	0.657	**$3.4x10^{-3}$***	0.874	0.453

OR: Odd Ratio; CI: Confidence Interval; *Significantly Associated

Table-4: Altered Motifs due to SNP variation

SNP	POS (hg 38)	LD (r^2)	LD (D')	Reference Allele	Altered Allele	Motif Changed	GENCODE genes	dbSNP func. annotation
rs4934	94614466	1	1	G	A	LXR, NF-E2, NrF-2, SMC3	SERPINA3	Missense
rs17473	94619305	1	1	C	G	BHLHE40, STAT	SERPINA3	Missense
rs754388	92649065	1	1	G	C	TCF12, TEF-1	RIN3	Intronic

Table-5: Haplotype analyses for two SNPs (rs754388, rs17473) by PLINK 1.9

Block	Haplotype	Case (frequency)	Control (frequency)	*p*-value[a]	Chi[2]	*p*-value [b]
Block 1						
H1	CC	0.732	0.647	**$9.0x10^{-4}$**	11.025	**0.0020**
H2	CT	0.206	0.279	**0.002**	9.351	**0.0100**
H3	TC	0.059	0.071	0.384	0.758	0.6900

p^a values calculated for multiple testing; p^b values are calculated after 1000 permutations; *significant at the $p<0.05$ level.

Table-S1: The chromosomal positions, relevant loci and associated functional roles along with global minor allele frequencies (MAF) and reference alleles of selected markers.

Gene	SNP	Position	Reference/ alternative Alleles	Orientation	Global MAF	Role/consequence
SERPINA1	rs4934	14:94614466	G/A	Forward	A=0.40	Missense variant
	rs1303	14:94378506	G/T	Forward	G=0.280	Missense variant
SERPINA3	rs17473	14:94619305	G/C/T	Forward	G=0.01	Missense variant
	rs1800463	14:94614674	T(germline)/C/A	Forward	C=0.01	Missense variant
RIN3	rs754388	14:92649065	C/G	Forward	G=0.118	Intron variant

Table-S2: Primer designed for amplification of selected SNPs by Primer3 software

ID	SNP	Seq 5'-3'	Length(bp)	Product size (bp)
S1F	rs4934-F	AAGCTAGCAAGAGGCAGCAG	20	362
S1R	rs4934-R	GATGCTCAGTGGGGAGAAGA	20	
S2F	rs1303-F	GGGACTCCAAGACAGGACAA	20	489
S2R	rs1303-R	CGACGAGAAAGGGACTGAAG	20	
S3F	rs1743-F	TATGAGGGACTCTGGGCACT	20	435
S3R	rs1743-R	CCTGGACATTGGTGAGACCT	20	
S4F	rs1800463-F	ACCCTAACAGCCCACTTGAC	20	412
S4R	rs1800463-R	TACAGCCTCTTGGCATCCTC	20	
S5F	rs754388-F	CCAGAGAAGATGTGCAGGGG	20	250
S5R	rs754388-R	CAGACTCTTCCTGTGGGTGA	20	

Table-S3: SBE probe sequence and their relevant lengths

IDs	Orientation	Sequence 5'-3'	Length
S1-SBE	F	CTGACTGACTGACTGACTTGGAGAGAATGTTACCTCTCCTG	45
S2-SBE	F	GACTGACTGACTGACTGACTGACTGACTAAGAGGGGAGACTTGGTATTTTG	55
S3-SBE	R	CTGACTGACTGACTGACTCTCGTCCCGGAAGTAAG	35
S4-SBE	F	GACTGACTGACTGACTGACTGACTGACTAAGAATGTCATCTTCTCCCCAC	50
S5-SBE	R	GACTGACTGACTGACTGACTGACTGACTGACTGACTGACTAACCCTTTAGCCTGGCATTC	60

Table-S4: Reagents used for Multiplex Reaction.

Reagent	Multiplex Reaction Volume(µl)
dNTPs mix (4mM)	1
Magnesium Chloride (25mM)	2.5
10x PCR Buffer	1
Taq Polymerase (5Units/µl)	0.4 (2Units)
Primer (Forward+Reverse) 8µM	4
Deionized Water	5.6
Purified DNA	0.5(12.5ng)
Total Volume	**15µl**

DISCUSSION

This study was designed to analyze the association of SNP variants, located on *RIN3*, *SERPINA1* and *SERPINA3* genes of chromosome 14, with COPD in the Pakistani population. Five SNPs residing on chromosome 14 (rs754388, rs4934, rs17473, rs1800463, and rs1303) were analyzed following genotyping analysis using different statistical tools including PLINK and Haploview.

Two of the SNPs (rs4934 and rs17473) were found to be independently and significantly associated with COPD in our studied population (Table-2). On applying logistic regression using PLINK v1.9, SNP rs4934 showed a strong association with the disease even when smoking and gender were taken as covariates (Table-3), while marker rs17473 showed an association with only gender as a covariate.

SNP rs4934, a missense variant located in an exonic region of the *SERPINA* gene, was also found to be significantly associated with disease under the recessive model of inheritance (*p*-value 0.0003; Table1). This SNP is known to alter the motifs NF-E2 motif as observed in HaploReg analysis (Table-4).[27] Two functional partners of this motif, haemoglobin beta and delta (HBB and HBD), are involved in the transportation of oxygen from the lungs to various peripheral tissues. A similar study performed on the Japanese population showed that Nrf2, a related factor of Nf-E2, is decreased in pulmonary macrophages.[28] The altered expression of this motif can be related to disease predisposition and needs to be confirmed by further studies.

Logistic regression analysis reveals a strong association of 'G' allele for rs4934 (*p*=0.04454; Odd Ratio: 1.233) with the disease. The association appears to get more significant on taking gender as a covariate (*p*=0.019; Odd Ratio=1.227). Similarly, an association of risk allele 'G' becomes even more significant in smoking individuals (*p*=5.13x10-7; Odd Ratio= 1.791) (Table-3). Previous studies have also shown higher COPD scores in smokers than in persons who quit smoking.[29,30]

rs17473, a missense mutation of the *SERPINA3* gene, showed a strong association under allelic, and genotypic models (Table-2). This SNP is known to affect *STAT* regulatory motif which mediates cellular responses to cytokines, interferon and growth factors.[31–33] Activation of the STAT pathway is involved in the development of pulmonary inflammatory disease and also acts as an activator of transcription in various genes that are mostly involved in B cell maturation and pathways related to immune systems.[34,35] A significant association of this marker (rs17473) with the disease in female patients is observed by taking gender as a covariate.

Although marker rs754388, located on *RIN3*, showed marginal association under the recessive model of inheritance (*p*-value of 0.05), it remained non-significantly associated when gender and smoking were taken as covariates. A case-control study done by genome-wide association strategy has also shown the allele 'C' of this marker to be significantly associated with COPD (*p*-value: 0.006, Odd Ratio: 1.51).[36] In previous studies, the upregulated expression of Rab5 and Rab7, the binding partners of *RIN3*, in smoking individuals suggested that these proteins might be associated with the repair process in tobacco-induced pulmonary damage.[37] Dysregulation caused by the risk allele of rs754388, on *RIN3*, thus, may lead to exacerbation of damage by smoking in COPD patients.

A pairwise linkage disequilibrium block of 1970 kb was constructed by using PLINK1.9 among all the associated SNPs in our study (Figure-1). rs754388 and rs17473 were observed to be in high linkage with each other. Thus, these SNPs may inherit together as a single block. Haplotype analysis, performed on SNPs that are in LD (rs754388 and rs17473), showed haplotype H1 to be strongly associated with the disease with a p-value of $9.0x10^{-4}$ while haplotype H2 was found to be more prevalent in controls than in cases with a *p*-value of $2x10^{-2}$ (Table-5). Thus, H1 is the risk haplotype whereas haplotype H2 might be a protective factor against the disease in our studied population.

CONCLUSION

Overall, the variants of *SERPINA1* and *SERPINA3* genes are significantly and independently associated with COPD in the local population of Pakistan. However, for further confirmation and designing of cost-effective, early-genetic diagnostics strategies, replication with a larger sample size and on different ethnic groups is necessary which may ultimately lead to the improvement of the quality of life of COPD patients.

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AUTHORS' CONTRIBUTION

The authors Sarooj Nadeem, Samra Kousar, Peerzada Fawad Ullah Jan, and Mariam Shahid contributed equally to this study.

Conflict of Interest

The authors declare that there is no conflict of interest.

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