Analysis of the association of ANO3/MUC15, COL4A4, RRB1, and KLK1 polymorphisms with COPD susceptibility in the Kashi population

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Abstract

Objective: Chronic obstructive pulmonary disease (COPD) is a complex, multifactorial, polygenic disease. The rate of occurrence of COPD in the Kashi population (Uyghur) is significantly higher than that observed nationwide. The identification of COPD-related genes in the Chinese Uyghur population could provide useful insights that could help us understand this phenomenon. Our previous whole-exome sequencing study of three Uyghur families with COPD demonstrated that 72 mutations in 55 genes might be associated with COPD; these included rs15783G > A in the anoctamin 3 (ANO3) gene/mucin 15 (MUC15) gene, rs1800517G > A in the collagen type IV alpha 4 chain (COL4A4) gene, rs11960G > A in the ribosome binding protein 1 (RRBP1) gene, and rs5516C > G in the kallikrein 1 (KLK1) gene. This case–control study aimed to further validate the association of the four mutations with COPD in the Chinese Uyghur population.

Methods: Sanger sequencing was used for the genotyping of four polymorphisms (ANO3/MUC15 rs15783, COL4A4 rs1800517, RRB1 rs11960, and KLK1 rs5516) in 541 unrelated Uyghur COPD patients and 534 Uyghur healthy controls. We then conducted stratified analyses based on the smoking status and airflow limitation severity, to explore the correlation between selected gene polymorphisms and COPD.

Results: ANO3/MUC15 rs15783 and KLK1 rs5516 polymorphisms could significantly reduce COPD risk (p < 0.05), but COL4A4 rs1800517 and RRB1 rs11960 polymorphisms were not correlated with COPD in the entire population. In a stratified analysis of smoking status, non-smokers with the ANO3/MUC15 rs15783G/G genotype (OR = 0.63, p = 0.032) or COL4A4 rs1800517 allele G (OR = 0.80, p = 0.023) had a reduced risk of COPD. Smokers with the RRB1 rs11960A/G genotype had a lower risk of COPD (OR = 0.41, p = 0.025). The KLK1 rs5516G > C polymorphism was associated with a decreased risk of COPD (OR < 1, p < 0.05), irrespective of the smoking status of individuals. No significant association with COPD severity was observed in individuals with these four polymorphisms (p > 0.05).

Conclusion: We identified four previously unreported mutations (ANO3/MUC15 rs15783, COL4A4 rs1800517, RRB1 rs11960, and KLK1 rs5516) that might decrease the COPD risk in individuals with different smoking statuses in the

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Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by persistent respiratory symptoms and airflow limitations [1], which are correlated with higher morbidity and mortality worldwide [2]. Cigarette smoking (CS) is the leading environmental risk factor for COPD; yet, even among heavy smokers, less than 50% develop COPD during their lifetime [3]. Epidemiologic studies have consistently shown that people who have never smoked might develop chronic airflow limitations [4]. In addition, during our previous epidemiological investigation in Kashi (Xinjiang, China), three three-generation families had presented with COPD [5]. The mechanisms responsible for the occurrence of these phenomena are unclear, but could depend at least partially on the genetic makeup of individuals. The epidemiological investigation also showed that the rate of occurrence of COPD in Chinese Uighurs whose age was above 40 years in Kashi was 17.01% [5] and was significantly higher than that observed nationwide [6]. The identification of COPD-related genes in the Chinese Uyghur population could provide useful insights that could help us understand this phenomenon.

Protein-coding genes constitute only ~1% of the human genome but harbor 85% of all mutations and significantly influence the occurrence of disease-related traits; thus, whole-exome sequencing (WES) can be used to obtain relevant insights into diverse human diseases [7]. Therefore, we performed WES on eight people with COPD and one healthy person from three Uyghur families with COPD in Kashi to screen for the susceptibility genes and polymorphisms related to COPD [8]. WES facilitated the identification of 72 single nucleotide variants (SNVs) of 55 genes, including g.26565254G > A (rs15783G > A, NC_000011.10) in the anoctamin 3 (ANO3) gene/mucin 15 (MUC15) gene, g.227051116G > A (rs1800517G > A, NC_000002.12) in the collagen type IV alpha 4 chain (COL4A4) gene, g.17619712G > A (rs11960G > A, NC_000020.11) in the ribosome binding protein 1 (RRBP1) gene, and g.50820217C > G (rs5516C > G, NC_000019.10) in the kallikrein 1 (KLK1) gene [8] (Fig. 1 and Additional files 1, 2, 3 and 4). Notably, the WES study evaluated only one healthy control as a reference; the screened 72 SNVs might include SNVs found in healthy people. Hence, it was necessary to verify the relationship between these SNVs and COPD in the Chinese Uyghur population. Our findings provide new light for the genetic risk factors associated with the occurrence of COPD.

Keywords: Chronic obstructive pulmonary disease, ANO3/MUC15, COL4A4, KLK1, RRBP1, Genetic polymorphism, Smoking status
72 SNVs and COPD in a case–control study. Moreover, although scholars have found that several gene polymorphisms might be related to COPD susceptibility [9, 10], the correlation between polymorphisms of the four genes (ANO3/MUC15 rs15783, COL4A4 rs1800517, RBBP1 rs11960, and KLK1 rs5516) and COPD risk is still poorly understood. Therefore, a case–control study of 1075 individuals (541 COPD patients and 534 healthy subjects) recruited from the same population was conducted, to evaluate the correlation between four selected SNVs and COPD risk. Furthermore, the correlations between the four selected SNVs and both clinical and functional parameters were explored. We found that the ANO3/MUC15, KLK1, COL4A4, and RBBP1 polymorphisms were closely associated with a decreased risk of COPD in individuals with different smoking statuses. The four SNVs were not found to be correlated with COPD severity after a stratified analysis based on airflow limitation severity. Our findings would provide further insights into the mechanism of occurrence of COPD.

Materials and methods
Experimental subjects
Individuals included 541 Uyghur unrelated COPD patients and 534 Uyghur healthy controls were consecutively recruited from the First people’s Hospital of Kashi during the period from 2018 to 2019. The age of all the participants was >40 years. Spirometry (Cosmed, Rome, Italy) was performed for all subjects. COPD was diagnosed in accordance with the diagnostic criteria outlined by the global initiative for chronic obstructive lung disease (GOLD), which specified that the post-bronchodilator forced expiratory volume in 1 s (FEV1)/forced vital capacity (FVC) should be <70% [1]. Baseline characteristics and classical COPD risk factors were recorded. None of the subjects were diagnosed with a history of atopy and an α1-antitrypsin deficiency. Additional inclusion and exclusion criteria for the participants were described by Gong et al. [11].

Informed written consent was obtained from all subjects. This study was carried out with the approval (Approval details: Kuai Shen Yan No. 70) of the Ethics Committee of the First People’s Hospital of Kashi. Five milliliters of peripheral blood samples were obtained from each of the 1075 participants for DNA extraction and transferred to BD Vacutainer ® EDTA-K2 blood collection tubes for DNA extraction.

Target gene sequencing
Genomic DNA samples were extracted from the peripheral blood using the Genomic DNA purification Kit (TIANGEN BIOTECH (BEIJING) CO., LTD, China), in accordance with the manufacturer’s instructions. The NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA) was used to measure the concentration and quality of extracted DNA. The four SNVs (ANO3/MUC15 rs15783, COL4A4 rs1800517, RBBP1 rs11960, and KLK1 rs5516) were genotyped via Sanger sequencing using a custom-by-design 48-Plex SNP scan Kit (Center for Genetic & Genomic Analysis, Genesky Biotechnologies Inc., Shanghai, China) [8]. The ABI3730XL (Applied Biosystems, USA) sequencer and GeneMapper 4.1 (Applied Biosystems, USA) were adopted to analyze the polymorphisms.

Statistical analysis
SPSS 18.0 statistical software (SPSS Inc., Chicago, IL, U.S.A.) was used for statistical analysis. Quantitative data were presented as means ± standard deviations (SD) for normally distributed values or medians plus interquartile ranges for non-normally distributed data. Categorical variables were described in terms of the count (%). Pearson’s chi-squared test was used to compare the differences in gender, smoking status, smoking index (SI), wood consumption, and coal consumption. The differences in age and body mass index (BMI) between different groups were assessed using an independent sample t-test, after quantile–quantile (QQ) plots demonstrated that these data showed an approximately normal distribution. FEV1%, FEV1/FVC, and annual household income values that did not conform to the normal distribution were calculated using the Mann–Whitney U test. Fisher’s exact test was used to assess the variations in all the SNVs frequencies by assessing the Hardy–Weinberg equilibrium (HWE). Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated to determine the association between the selected SNVs and COPD risk using logistic regression analysis, with adjustments for age, gender, and BMI. Using PLINK software (version 2.0), the effects attributable to single nucleotide polymorphisms (SNPs) were fitted under five models of inheritance, i.e., the additive, codominant, dominant, recessive, and allele models. The stratified model was applied for subgroup analysis after taking the smoking status and pulmonary function of participants into consideration. We used p < 0.05 as the cut-off value for determining statistical significance.

Results
Characteristics of the study population
The clinical characteristics and spirometry data of 1075 participants in the case–control study have been provided in Table 1. A total of 541 COPD patients (280 male and 261 female) and 534 controls (234 male and 300 female) were included in the study. There was no significant difference between COPD cases and controls
in terms of wood consumption, coal consumption, and annual household income ($p > 0.05$). The differences between the two groups with regard to age, gender, BMI, smoking status, smoking index, FEV1%, and FEV1/FVC were statistically significant ($p < 0.05$). Within the COPD population, 150, 292, 82, and 17 patients had mild disease (FEV1 ≥ 80% predicted), moderate disease (50% ≤ FEV1 < 80% predicted), severe disease (30% ≤ FEV1 < 50% predicted), and very severe disease (FEV1 < 30% predicted), respectively.

### Table 1 General characteristics of COPD patients and healthy subjects

| Variables                                      | Case (n = 541) | Control (n = 534) | p   |
|------------------------------------------------|----------------|-------------------|-----|
| Age, years (mean ± SD)                         | 61.11 ± 12.26  | 54.86 ± 10.73     | <0.001 |
| Gender (male/female), n                        | 280/261        | 234/300           | 0.009 |
| BMI (kg/m²) (mean ± SD)                        | 23.56 ± 4.22   | 25.55 ± 4.17      | <0.001 |
| Annual household income (CNY, yuan) (median, range) | 16,939 (10,294–23,442) | 15,298 (9762–23,057) | 0.197 |
| Smoking status (never/former/current), n       | 417/23/101     | 465/17/52         | <0.001 |
| Smoking index ($< 1/1 ≤ SI ≤ 400/400 < SI ≤ 800/SI > 800$), n | 417/ 98/16/10  | 465/52/11/6       | <0.001 |
| Coal consumption (yes/no), n                   | 513/28         | 516/18            | 0.144 |
| Wood consumption (yes/no), n                   | 519/22         | 500/34            | 0.09  |
| Time-to-first respiratory symptoms$^b$, years ($≤ 5/5 < Time ≤ 10/ > 10$), n | 196/277/68     | –                 | –     |
| GOLD grade (1/2/3/4), n                        | 150/292/82/17   | –                 | –     |
| Domiciliary oxygen therapy (nasal catheter/NIV/no), n | 243/107/191     | –                 | –     |
| Pulmonary drugs (inhaled), n                   | 11/5/79        | –                 | –     |
| Combination therapy (LABA + LAMA/ICS + LABA/ICS + LAMA + LABA) | 85/138/30      | –                 | –     |
| Other (no/unavailable)                         | 169/24         | –                 | –     |
| Pulmonary drugs (oral), n                      | 174            | –                 | –     |
| Theophylline                                   | 197            | –                 | –     |
| LTRA                                           | 139/36         | –                 | –     |
| Lung function (median, range)                  | 69.00 (53.92–82.00) | 86.00 (74.00–98.00) | <0.001 |
| FEV1%                                          | 0.62 (0.55–0.66) | 0.80 (0.75–0.86)  | <0.001 |

FEV1: Forced expiratory volume in 1 s, FVC: Forced vital capacity, BMI: Body mass index, NIV: Noninvasive ventilation, SABA: Short-acting beta2-agonist, SAMA: Short-acting muscarinic antagonist, LABA: Long-acting beta2-agonist, LAMA: Long-acting muscarinic antagonist, ICS: Inhaled corticosteroids, LTRA: Leukotriene receptor antagonist, GOLD: Global initiative for chronic obstructive lung disease

$^a$ Smoking index = number x year

$^b$ Respiratory symptoms, including cough, sputum production, and dyspnea

### Genotype analyses of COPD risk

Four SNVs (ANO3/MUC15 rs15783, COL4A4 rs1800517, RRBP1 rs11960, and KLK1 rs5516) of target genes were successfully genotyped in the case–control study. The call success rate was more than 99%. Genotype distributions of the above SNVs were in accordance with Hardy–Weinberg equilibrium predictions ($p > 0.05$) (Table 2).

To identify whether the polymorphisms of these genes were related to COPD susceptibility, we analyzed five genetic models (additive, codominant, dominant,

### Table 2 Basic information and allele frequencies among all SNVs

| Gene         | SNV ID | Chromosome Position | Common Allele | Minor Allele | MAF Cases | MAF Controls | HWE |
|--------------|--------|---------------------|---------------|--------------|-----------|--------------|-----|
| ANO3/MUC15   | rs15783| Chr11: 26,586,801   | A             | G            | 0.382     | 0.410        | 0.339 |
| COL4A4      | rs1800517| Chr2: 227,915,832  | A             | G            | 0.473     | 0.501        | 0.803 |
| RRBP1       | rs11960| Chr20: 17,600,357   | A             | G            | 0.437     | 0.425        | 0.160 |
| KLK1        | rs5516 | Chr19: 51,323,473   | G             | C            | 0.266     | 0.301        | 0.114 |

SNV: Single nucleotide variant, MAF: Minor allele frequency, HWE: Hardy–Weinberg equilibrium
recessive, and allele) that were adjusted for age, gender, and BMI (Table 3). The results showed that the ANO3/MUC15 rs15783G>A and KLK1 rs5516C>G polymorphisms were correlated with a decreased risk of COPD ($p < 0.05$). Individuals with the ANO3/MUC15 rs15783 G/G genotype had a reduced risk of COPD.

### Table 3 Analysis of genotypes of ANO3/MUC15 rs15783, COL4A4 rs1800517, RRBP1 rs11960, and KLK1 rs5516

| SNV ID         | Model        | Genotype | Case N (%) | Control N (532) | OR(95%CI) a      | p a     | OR(95%CI) b       | p b     |
|----------------|--------------|----------|------------|-----------------|-----------------|---------|-----------------|---------|
| ANO3/MUC15 rs15783 | Codominant   | A/A      | 203        | 196             | 1.07(0.82–1.39) | 0.628   | 1.01(0.77–1.34) | 0.932   |
|                |              | A/G      | 262        | 237             | 0.74(0.52–1.05) | 0.089   | 0.67(0.46–0.98) | 0.038   |
|                |              | G/G      | 76         | 100             |                  |         |                  |         |
|                | Dominant     | A/A      | 203        | 196             | 0.97(0.76–1.24) | 0.799   | 0.91(0.70–1.18) | 0.476   |
|                |              | A/G, A/G | 338        | 337             |                  |         |                  |         |
|                | Recessive    | A/A, A/G | 465        | 433             |                  |         |                  |         |
|                |              | G/G      | 76         | 100             | 0.71(0.51–0.98) | 0.037   | 0.67(0.47–0.94) | 0.021   |
|                | Additive     | –        | –          | –               | 0.90(0.75–1.06) | 0.202   | 0.86(0.71–1.02) | 0.087   |
|                | Allele       | A        | 668        | 629             | 0.89(0.75–1.06) | 0.196   | 0.85(0.71–1.02) | 0.082   |
|                |              | G        | 414        | 437             |                  |         |                  |         |
| COL4A4 rs1800517 | Codominant   | A/A      | 157        | 137             | 0.86(0.65–1.15) | 0.313   | 0.81(0.60–1.10) | 0.173   |
|                |              | A/G      | 255        | 258             | 0.81(0.58–1.13) | 0.212   | 0.75(0.53–1.07) | 0.114   |
|                | Dominant     | A/A      | 128        | 138             | 0.84(0.65–1.10) | 0.216   | 0.79(0.60–1.05) | 0.104   |
|                |              | A/G, A/G | 383        | 396             |                  |         |                  |         |
|                | Recessive    | A/A, A/G | 412        | 395             | 0.89(0.68–1.17) | 0.407   | 0.86(0.64–1.16) | 0.319   |
|                | Additive     | –        | –          | –               | 0.90(0.76–1.06) | 0.208   | 0.87(0.73–1.03) | 0.109   |
|                | Allele       | A        | 569        | 532             | 0.90(0.76–1.06) | 0.198   | 0.86(0.72–1.03) | 0.102   |
|                |              | G        | 511        | 534             |                  |         |                  |         |
| RRBP1 rs11960  | Codominant   | A/A      | 170        | 179             | 1.11(0.84–1.45) | 0.463   | 1.04(0.79–1.39) | 0.789   |
|                |              | A/G      | 267        | 254             | 1.09(0.77–1.54) | 0.646   | 1.00(0.69–1.44) | 0.992   |
|                | Dominant     | A/A      | 170        | 179             | 1.10(0.85–1.42) | 0.462   | 1.03(0.79–1.35) | 0.841   |
|                |              | A/G, A/G | 369        | 353             |                  |         |                  |         |
|                | Recessive    | A/A, A/G | 437        | 433             | 1.02(0.75–1.39) | 0.895   | 0.98(0.71–1.35) | 0.877   |
|                | Additive     | –        | –          | –               | 1.05(0.89–1.25) | 0.573   | 1.00(0.84–1.20) | 0.962   |
|                | Allele       | A        | 607        | 612             | 1.05(0.89–1.25) | 0.572   | 1.00(0.84–1.20) | 0.962   |
|                |              | G        | 471        | 452             |                  |         |                  |         |
| KLK1 rs5516    | Codominant   | G/G      | 289        | 269             | 0.97(0.75–1.25) | 0.800   | 0.98(0.75–1.28) | 0.890   |
|                |              | G/C      | 210        | 202             | 0.61(0.39–0.99) | 0.028   | 0.62(0.39–0.98) | 0.042   |
|                | Dominant     | G/G      | 289        | 269             | 0.89(0.70–1.13) | 0.332   | 0.90(0.70–1.16) | 0.417   |
|                |              | C/C, G/C | 248        | 260             |                  |         |                  |         |
|                | Recessive    | G/G, G/C | 499        | 471             | 0.62(0.40–0.95) | 0.028   | 0.62(0.40–0.98) | 0.039   |
|                | Additive     | –        | –          | –               | 0.85(0.71–1.02) | 0.087   | 0.86(0.71–1.04) | 0.126   |
|                | Allele       | G        | 788        | 740             | 0.85(0.70–1.02) | 0.079   | 0.85(0.70–1.04) | 0.118   |
|                |              | C        | 286        | 318             |                  |         |                  |         |

*a p values were calculated by logistic regression analysis

*b p values were calculated by logistic regression analysis, after adjusting for age, gender, and BMI

The values of p and OR (95%CI), along with the statistical significance, are highlighted in bold

SNV: Single nucleotide variation, OR: Odds Ratio, 95%CI: 95% confidence interval
as shown by the results obtained with the codominant model (OR = 0.67, p = 0.038) and recessive model (OR = 0.67, p = 0.021). The KLK1 rs5516 C/C genotype was associated with a decreased risk of COPD under the codominant model (OR = 0.62, p = 0.042) and recessive model (OR = 0.62, p = 0.039). No significant association was observed for other SNVs.

Stratified analysis of rs15783 G > A, rs1800517 G > A, rs11960 G > A, and rs5516 C > G in the case–control study based on smoking status

COPD is a complex disease that is likely influenced by environmental factors, multiple genes, and gene-by-smoking/environmental interactions [12, 13]. To investigate whether the association between these four variants and COPD risk differed between smokers and non-smokers, we conducted a subgroup analysis (Table 4).

We found that ANO3/MUC15 rs15783 and COL4A4 rs1800517 polymorphisms were only related to an altered risk of COPD in non-smokers (p < 0.05). Individuals with the ANO3/MUC15 rs15783 G/G genotype had a reduced risk in the codominant (OR = 0.63, p = 0.032) and recessive (OR = 0.63, p = 0.017) models. Another COL4A4 rs1800517 G > A polymorphism also played a protective role in non-smokers, and carriers of the minor allele (G) had a lower risk for COPD, based on the results for the allele (OR = 0.80, p = 0.023) and additive (OR = 0.80, p = 0.025) models. Additionally, the COL4A4 rs1800517 G/G genotype was also associated with a decreased risk of COPD in the codominant (OR = 0.64, p = 0.026) and dominant (OR = 0.72, p = 0.039) models.

The RRBP1 rs11960 G > A played a protective role in smokers (p < 0.05). The RRBP1 rs11960 A/G genotype was associated with a decreased risk of COPD, based on the results obtained with the codominant (OR = 0.41, p = 0.025) and dominant (OR = 0.40, p = 0.018) models. The RRBP1 rs11960 G > A SNV fitted the additive model, and a significantly decreased risk of COPD was observed in the presence of a “G” allele (OR = 0.62, p = 0.041).

We observed that the KLK1 rs5516 C > G polymorphism influenced COPD risk in both smokers and non-smokers in different genetic models. The results of the stratified analyses for the recessive model (OR = 0.60, p = 0.046) indicated that the KLK1 rs5516 C/C genotype was associated with a decreased risk of COPD in non-smoking participants. However, we also found that the KLK1 rs5516 polymorphism was strongly related to COPD risk in smoking participants, while carriers of the G/C genotype were at lower risk for COPD, based on the results obtained with the codominant (OR = 0.39, p = 0.007) and dominant (OR = 0.41, p = 0.007) models. The KLK1 rs5516 C > G SNV fitted the additive model, and was observed to be associated with a significantly decreased risk of COPD in the presence of a "C" allele (OR = 0.56, p = 0.024).

Stratified analysis of rs15783 G > A, rs1800517 G > A, rs11960 G > A, and rs5516 C > G based on COPD severity in the case–control study

Using a logistic regression model, we investigated the association of ANO3/MUC15, COL4A4, RRBP1, and KLK1 genotype distributions and disease severity based on lung function, using a cutoff limit of FEV1 = 50% predicted. As shown in Table 5, no significant correlation was found between the genotype distribution of ANO3/MUC15 rs15783 > G, COL4A4 rs1800517 > A, RRBP1 rs11960 > A, and KLK1 rs5516 C > G polymorphisms and airflow limitation severity (p > 0.05).

Discussion

COPD is a heterogeneous and complex disease, and the genetic composition of an individual could significantly determine susceptibility [3–5]. We first screened four mutations, namely, ANO3/MUC15 rs15783, COL4A4 rs1800517, RRBP1 rs11960, and KLK1 rs5516, which were identified to be associated with COPD via a WES analysis of a cluster of three COPD families. The results of association analysis for these four SNVs indicated that the ANO3/MUC15 rs15783 and KLK1 rs5516 polymorphisms were associated with COPD susceptibility in the entire population. Further, upon performing stratified analysis, we found that the ANO3/MUC15, KLK1, COL4A4, and RRBP1 polymorphisms were closely associated with a reduced risk of COPD in individuals with different smoking statuses, but were not related to disease severity.

The ANO3/MUC15 rs15783, COL4A4 rs1800517, RRBP1 rs11960, and KLK1 rs5516 are non-synonymous variants (via NCBI database: https://www.ncbi.nlm.nih.gov/). Three of the 4 SNVs (COL4A4 rs1800517, RRBP1 rs11960, and KLK1 rs5516) are exonic SNPs and correspond to a missense substitution. Only SNV rs15783 occurs in the overlapping genes ANO3 and MUC15; it occurs as an intronic SNP in ANO3 (intron 13 of NM_031418.2 transcript) and exonic SNP in MUC15, and corresponds to a missense substitution (Thr > Ile) at position 229 in the MUC15 isoform b [14]. The enrichment analyses of the Gene Ontology (GO) database (http://www.geneontology.org) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway (https://www.genome.jp/kegg) indicated that the four genes did not occur in the same KEGG metabolic pathway. In addition, protein interaction network analysis using the STRING database (https://string-db.org/) showed that these proteins did not interact with each other in any manner.
Prolonged exposure to CS might increase the total burden attributable to environmental factors such as genetic predisposition, which might increase the risk of COPD sufficiently and cause COPD [12, 13], especially in individuals with high levels of susceptibility. In the present study, we first demonstrated that the ANO3/MUC15 rs15783 and COL4A4 rs1800517 SNVs were associated with a reduced risk of COPD in non-smokers. Notably,

Table 4  Analysis of genotypes of all SNVs among non-smoker and smoker group

| SNV ID       | Model     | Genotype | Non-smoking | Smoking |
|--------------|-----------|----------|-------------|---------|
|              |           |          | Case Control | OR(95%CI) | p      | Case Control | OR(95%CI) | p      |
|              | A/A       | A/A      | 158 171 1   | 45 25 1  |        |            |            |        |
|              | A/G       | A/G      | 203 206     | 59 31 1  | 1.12(0.55–2.26) | 0.754 |
|              | G/G       | G/G      | 56 88 0.63(0.42–0.96) | 0.032 | 20 12 0.90(0.36–2.25) | 0.817 |
|              | Dominant  | A/A      | 158 171 1   | 45 25 1  |        |            |            |        |
|              |           | G/G, A/G | 259 294 0.90(0.67–1.19) | 0.450 | 79 43 1.05(0.55–2.03) | 0.878 |
|              | A/G       | G/G      | 56 88 0.63(0.43–0.92) | 0.017 | 20 12 0.84(0.37–1.94) | 0.689 |
|              | Additive  | –        | – 0.84(0.68–1.02) | 0.075 | – – 0.98(0.62–1.53) | 0.914 |
|              | Allele    | A        | 519 548 1   | 149 81 1 |        |            |            |        |
|              |           | G        | 315 382 0.83(0.68–1.02) | 0.071 | 99 55 0.98(0.62–1.54) | 0.913 |
|              |            |          |            |        |        |            |            |        |
|              | A/A       | A/A      | 125 115 1   | 32 22 1  |        |            |            |        |
|              | A/G       | A/G      | 197 226 0.76(0.55–1.06) | 0.110 | 58 32 1.16(0.56–2.46) | 0.691 |
|              | G/G       | G/G      | 95 124 0.64(0.44–0.95) | 0.026 | 33 14 1.86(0.76–4.56) | 0.174 |
|              | Dominant  | A/A      | 125 115 1   | 32 22 1  |        |            |            |        |
|              |           | G/G, A/G | 292 350 0.72(0.53–0.98) | 0.039 | 91 46 1.36(0.68–2.74) | 0.388 |
|              | A/G       | G/G      | 95 124 0.77(0.55–1.06) | 0.108 | 33 14 1.69(0.79–3.64) | 0.178 |
|              | Additive  | –        | – 0.80(0.66–0.97) | 0.025 | – – 1.35(0.87–2.10) | 0.182 |
|              | Allele    | A        | 447 456 1   | 122 76 1 |        |            |            |        |
|              |           | G        | 387 474 0.80(0.65–0.97) | 0.023 | 124 60 1.37(0.87–2.15) | 0.169 |
|              |            |          |            |        |        |            |            |        |
|              | A/A       | A/A      | 128 165 1   | 42 14 1  |        |            |            |        |
|              | A/G       | A/G      | 212 215 1.21(0.88–1.65) | 0.242 | 55 39 0.41(0.19–0.89) | 0.025 |
|              | G/G       | G/G      | 75 84 1.14(0.76–1.72) | 0.521 | 27 15 0.39(0.15–1.01) | 0.053 |
|              | Dominant  | A/A      | 128 165 1   | 42 14 1  |        |            |            |        |
|              |           | G/G, A/G | 287 299 1.19(0.88–1.60) | 0.254 | 82 54 0.40(0.19–0.85) | 0.018 |
|              | A/G       | G/G      | 75 84 1.02(0.71–1.47) | 0.098 | 27 15 0.71(0.33–1.53) | 0.385 |
|              | Additive  | –        | – 1.09(0.89–1.33) | 0.407 | – – 0.62(0.39–0.98) | 0.041 |
|              | Allele    | A        | 468 545 1   | 139 67 1 |        |            |            |        |
|              |           | G        | 362 383 1.09(0.89–1.33) | 0.406 | 109 69 0.62(0.39–0.98) | 0.039 |
|              |            |          |            |        |        |            |            |        |
|              | A/A       | A/A      | 213 240 1   | 76 29 1  |        |            |            |        |
|              | A/G       | A/G      | 172 170 1.18(0.88–1.59) | 0.267 | 38 32 0.39(0.19–0.77) | 0.007 |
|              | G/C       | G/C      | 29 52 0.64(0.38–1.08) | 0.096 | 9 6 0.51(0.16–1.70) | 0.276 |
|              | Dominant  | A/A      | 213 240 1   | 76 29 1  |        |            |            |        |
|              |           | C/C, G/C | 201 222 1.06(0.80–1.40) | 0.697 | 47 38 0.41(0.21–0.78) | 0.007 |
|              | A/G       | G/G      | 385 410 1   | 114 61 1 |        |            |            |        |
|              | Additive  | –        | – 0.94(0.76–1.16) | 0.557 | – – 0.56(0.34–0.93) | 0.024 |
|              | Allele    | G        | 598 650 1   | 190 90 1 |        |            |            |        |
|              |           | C        | 230 274 0.94(0.75–1.16) | 0.549 | 56 44 0.55(0.33–0.91) | 0.020 |

*p values were calculated by logistic regression analysis, after adjusting for age, gender, and BMI
The values of *p* and OR (95% CI), along with the statistical significance, are highlighted in bold
SNV Single nucleotide variation, OR Odds Ratio, 95% CI 95% confidence interval
smokers did not have a significantly altered risk of COPD, regardless of the genetic model, compared to non-smokers. This suggests that the G/G genotype of ANO3/MUC15 rs15783 and allele G of COL4A4 rs1800517 might exhibit only a weak protective effect against COPD in non-smokers; this protective relationship would be reversed if non-smokers carrying protective mutations at these two loci were affected by CS. Here, the RRBP1

| SNV ID       | Model          | Genotype | FEV1 predicted, n | OR(95%CI) | p      |
|--------------|----------------|----------|-------------------|-----------|--------|
| ANO3/MUC15 rs15783 | Codominant | A/A      | 167               | 1         |        |
|              | A/G            | 210      | 0.88(0.55–1.41)   | 0.584     |        |
|              | G/G            | 65       | 1.29(0.62–2.70)   | 0.497     |        |
|              | Dominant       | A/A      | 167               | 1         |        |
|              | G/G, A/G       | 275      | 0.95(0.60–1.49)   | 0.819     |        |
|              | Recessive      | A/A, A/G | 377               | 1         |        |
|              | G/G            | 65       | 1.39(0.71–2.76)   | 0.340     |        |
|              | Additive       | –        | –                 | –         |        |
|              | Allele         | A        | 544               | 124       | 1      |
|              | G              | 340      | 74                | 1.05(0.77–1.45) | 0.746 |
| COL4A4 rs1800517 | Codominant | A/A      | 125               | 32        | 1      |
|              | A/G            | 213      | 1.33(0.80–2.22)   | 0.276     |        |
|              | G/G            | 103      | 1.08(0.60–1.95)   | 0.792     |        |
|              | Dominant       | A/A      | 125               | 32        | 1      |
|              | G/G, A/G       | 316      | 1.24(0.77–1.98)   | 0.376     |        |
|              | Recessive      | A/A, A/G | 338               | 74        | 1      |
|              | G/G            | 103      | 0.91(0.55–1.51)   | 0.720     |        |
|              | Additive       | –        | –                 | –         |        |
|              | Allele         | A        | 463               | 106       | 1      |
|              | G              | 419      | 92                | 1.06(0.78–1.44) | 0.725 |
| RRBP1 rs11960 | Codominant    | A/A      | 134               | 36        | 1      |
|              | A/G            | 221      | 1.33(0.81–2.17)   | 0.259     |        |
|              | G/G            | 85       | 1.40(0.74–2.65)   | 0.306     |        |
|              | Dominant       | A/A      | 134               | 36        | 1      |
|              | G/G, A/G       | 306      | 1.34(0.85–2.14)   | 0.207     |        |
|              | Recessive      | A/A, A/G | 355               | 82        | 1      |
|              | G/G            | 85       | 1.18(0.66–2.10)   | 0.570     |        |
|              | Additive       | –        | –                 | –         |        |
|              | Allele         | A        | 489               | 118       | 1      |
|              | G              | 391      | 80                | 1.20(0.88–1.65) | 0.250 |
| KLK1 rs5516  | Codominant    | G/G      | 231               | 58        | 1      |
|              | G/C            | 176      | 1.30(0.82–2.08)   | 0.278     |        |
|              | C/C            | 31       | 1.19(0.49–2.85)   | 0.702     |        |
|              | Dominant       | G/G      | 231               | 58        | 1      |
|              | C/C, G/C       | 207      | 1.28(0.82–2.00)   | 0.279     |        |
|              | Recessive      | G/G, G/C | 407               | 92        | 1      |
|              | C/C            | 31       | 1.07(0.45–2.52)   | 0.877     |        |
|              | Additive       | –        | –                 | –         |        |
|              | Allele         | G        | 638               | 150       | 1      |
|              | C              | 238      | 48                | 1.18(0.83–1.70) | 0.356 |

P values were calculated by logistic regression analysis, after adjusting for age, gender, and BMI
FEV1 Forced expiratory volume in 1 s, SNV Single nucleotide variation, OR Odds Ratio, 95% CI 95% confidence interval
rs11960 polymorphism was also first shown to be associated with COPD susceptibility in smokers; an individual with allele G could have a significantly reduced risk of COPD. Interestingly, we observed that this protective effect of RBBP1 rs11960 could be observed only in those carrying heterozygous mutations. In addition, we found that the risk of COPD was decreased in smokers with the KLK1 rs5516 G/C genotype and non-smokers with the rs5516 C/C variant, though individuals with different smoking statuses were affected by homozygous and heterozygous mutations.

COPD manifests as persistent airflow obstruction. Although mucus and other epithelial secretions of the airway play a critical role in protecting the lung during acute injury, impaired mucus clearance after chronic mucus hyperproduction causes airway obstruction, infection, and inflammation, which contribute to morbidity in common pulmonary disorders, including chronic obstructive pulmonary disease and asthma [15]. ANO3 (also known as TMEM16C) belongs to the TMEM16 family encoding predicted membrane proteins that are involved in the functioning of intracellular calcium-activated chloride channels (CaCCs). These proteins perform many important functions in cell physiology, including the facilitation of fluid secretion from acinar cells of secretory glands, regulation of neuronal excitability, and regulation of smooth muscle contraction [16]. Although limited information is available regarding the function of TMEM16C in COPD pathology, another anoctamin (TMEM16A) has been shown to be involved in CaCC-based mechanisms resulting in excessive mucus secretion and airway smooth muscle contraction during inflammatory airway disease [17]. The process of GO annotation of genes related to TMEM16A and TMEM16C includes genes that facilitate intracellular CaCC activity, suggesting that TMEM16C might also be involved in the pathophysiological mechanism of occurrence of COPD, via the regulation of mucus secretion or airway smooth muscle contraction. However, studies on ANO3 have mainly focused on genetic dystonia [18]. MUC15 (Mucin 15, cell surface associated) belongs to the family of mucin genes, which encode large epithelial glycoproteins that are major constituents of the mucus that covers the surfaces of epithelial tissues and provides a physical barrier that protects the underlying epithelium [19]. MUC15 is a highly glycosylated protein exhibiting the structural features observed in other intraline membrane mucins [20]. Therefore, we hypothesized that MUC15 might play a key role in the occurrence and development of COPD. However, studies on the correlation between MUC15 and human disease mainly focus on malignant tumors [21], and its correlation with COPD is still poorly understood. We showed that ANO3/MUC15 rs15783 might act as a protective factor against COPD. It should be noted that both ANO3 and MUC15, which are affected by the SNV rs15783, may be related to COPD; thus, ANO3/MUC15 rs15783 SNV is highly likely to alter the risk of COPD.

COL4A4 encodes one of the six subunits of type IV collagen, the major structural component of basement membranes that majorly contribute to the strength of the blood-gas barrier (BGB) [22]. The BGB (also known as the alveolar-capillary barrier) is the key functional element of the lung, and serves as the site of oxygen and carbon dioxide exchange between distal airspaces and the pulmonary vasculature [23]. A recent animal (mouse and chick) study found that type IV collagen plays a key role during alveolar morphogenesis and is critical for the proper formation of the BGB and the process of septation [22]. COPD is characterized by persistent respiratory symptoms and airflow limitations caused by airway and/or alveolar abnormalities that are influenced by a host of factors, including abnormal lung development [1]. An impaired microvascular barrier also has been observed in COPD patients [24, 25]. Meanwhile, it has been reported that type IV collagen is important for maintaining the integrity of the vascular basement membrane [26]. The important role of type IV collagen in the alveolar sphere and pulmonary vascular basement membrane structure suggests that a mutation in COL4A4 might contribute to COPD occurrence. Although COL4A4 is widely expressed in both kidney (RPKM 5.3) and lung (RPKM 4.3) tissues (via NCBI database: https://www.ncbi.nlm.nih.gov/), previous studies have mainly focused on COL4A4 expression in the glomerular basement membrane [27, 28]. The specific role of COL4A4 in COPD development remains unknown. Our study has shown for the first time that COL4A4 rs1800517 allele G may act as a protective factor against COPD. We speculated that the COL4A4 rs1800517 SNV might prevent the occurrence of COPD as it affects basement membrane components in the lung tissue.

Endoplasmic reticulum stress (ERS) can be attributed to the improper folding or conformation (misfolded protein) of proteins, which can interfere with the normal physiological functions of the cell [29]. The unfolded protein response (UPR) is a mechanism by which cells control ER protein homeostasis [30]. RBBP1 was originally identified as a ribosome-binding protein located on the rough endoplasmic reticulum, and was majorly involved in regulating the secretion of intracellular proteins and alleviation of ERS [31–33]. ERS can be strongly induced by cigarette smoke extracts [34]. Several studies have indicated that the inhibition of ERS can largely ameliorate CS-induced airway inflammation and emphysema through the suppression of inflammation, apoptosis, and oxidative stress, via the...
SNV might be associated with COPD susceptibility. While RRB1P is associated with the regulation of UPR, its role in RRB1P in COPD development is unclear. Here, the **RRBP1 rs11960** polymorphism was first shown to be a protective factor in smokers. Therefore, we speculated that RRB1P might potentially exhibit protective effects against COPD by inhibiting CS-induced ERS.

A characteristic pathologic feature of COPD is a protease-antiprotease imbalance [39]. **KLK1** is one of the important regulatory genes contributing to a protease-antiprotease imbalance, and is essential for the pathogenesis of COPD. Previous studies have shown that the uncontrolled activity of KLK1 can lead to the direct proteolytic cleavage of the pro-epidermal growth factor, release of mature epidermal growth factor, and subsequent activation of epidermal growth factor receptor (EGFR), thereby resulting in metaplasia and excessive mucus secretion in individuals with airway diseases [40]. In addition, EGFR activation was also related to the pathologic phenotypes of epithelial cells in smokers [41]. Upon exposure to the reactive oxygen species derived from cigarette smoke or endogenous sources, the up-regulation of KLK1 activity leads to the degradation of the hyaluronic acid (HA) occurring on airway epithelial cells [42], subsequently weakening the limiting effect of the proteolytic activity of HA [43]. The **KLK1 rs5516** polymorphism influences susceptibility of multiple diseases and conditions, including aortic aneurysm [44], thoracic aortic dissection [45], SLE with nephritis [46], and essential hypertension [47]. To date, this is the first study to demonstrate that **KLK1 rs5516** SNV might be associated with COPD susceptibility.

We used a combination of whole-exome and targeted sequencing to search for novel genetic characteristics of COPD in the Chinese Uyghur population. To our knowledge, we are the first to report the association between the four SNVs and COPD risk. However, several limitations are associated with our study. First, our population verification process is based on the ethnic characteristics of individuals from Kash, i.e., the Uyghur population. Therefore, the results are not representative of other ethnic groups. Second, the sample size of the subgroup of smokers was small; hence, our findings need to be confirmed in studies with a larger sample size of smokers. In addition, although polymorphisms of four genes identified in this study might be associated with lower prevalence of COPD, the specific role of these genes in COPD pathogenesis is unclear. Additional investigations, including multi-omics approaches and functional studies are needed, to further determine disease causality and biological mechanisms of action of these genes.

**Conclusion**

The present study identified four previously unreported mutations that were associated with COPD susceptibility in individuals with different smoking statuses. The **ANO3/MUC15 rs15783G > A** and **COL4A4 rs1800517G > A** polymorphisms had protective effects on non-smokers, while the **RRBP1 rs11960G > A** polymorphism had protective effects on smokers. The **KLK1 rs5516C > G** polymorphism was associated with a decreased risk of COPD, irrespective of whether the individual was a smoker or non-smoker. Our findings provide new insights regarding the contribution of the genetic makeup of an individual to COPD pathogenesis in a Chinese Uyghur population.

**Abbreviations**

COPD: Chronic obstructive pulmonary disease; **ANO3/MUC15**: Anoctamin 3/Mucin 15; **COL4A4**: Collagen type IV Alpha 4 chain; **KLK1**: Kallikrein 1; **RRBP1**: Ribosome binding protein 1; CS: Cigarette smoking; WES: Whole-exome sequencing; SNVs: Single nucleotide variants; GOLD: Global initiative for chronic obstructive lung disease; FEV1: Forced expiratory volume in 1 s; FVC: Forced vital capacity; BMI: Body mass index; SPN: Single nucleotide polymorphism; MAF: Minor allele frequency; HWE: Hardy–Weinberg equilibrium; QQ: Quantile–quantile; SD: Standard deviation; ORs: Odds ratios; CIs: Confidence intervals; CaCCs: Calcium-activated chloride channels; GO: Gene ontology; BG: Blood-gas barrier; ERS: Endoplasmic reticulum stress; UPR: The unfolded protein response; EGFR: Epidermal growth factor receptor; NIV: Noninvasive ventilation; SABA: Short-acting beta2-agonist; SAMA: Short-acting muscarinic antagonist; LABA: Long-acting beta2-agonist; LAMA: Long-acting muscarinic antagonist; ICS: Inhaled corticosteroids; LT: Leukotriene receptor antagonist.

**Supplementary Information**

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**Author contributions**

LT and XZ drafted the work or revised it critically for important content; LT, XZ, MT, TM, JR, CX, AZ, AA and PA performed the experiments and collected data; LT, HG and SY analyzed the data; LT and HG prepared the figures and tables; LL conceived and designed the experiments. All authors read and approved the final manuscript.

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