Research Paper

A Functional Variant rs6435156C>T in *BMPR2* is Associated With Increased Risk of Chronic Obstructive Pulmonary Disease (COPD) in Southern Chinese Population

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ABSTRACT

Backgrounds: Bone morphogenetic protein receptor type 2 (BMPR2) signaling is anti-inflammatory. Decreased BMPR2 expression was seen in lung tissue from chronic obstructive pulmonary disease (COPD) patients.
Methods: The selected single nucleotide polymorphisms (SNPs) in *BMPR2* were genotyped with polymerase chain reaction (PCR) ligase detection reaction. The effects of SNPs on gene expression were analyzed with luciferase assays. The mRNA and protein expression levels of BMPR2 in peripheral blood mononuclear cells (PBMCs) from COPD patients were determined by quantitative PCR and western blotting, respectively.
Findings: Two SNPs, rs6435156C>T and rs1048829G>T in the 3′-untranslated region (3′UTR) of *BMPR2* were selected and genotyped in COPD case and healthy control subjects from southern Chinese population. Both of them were found associated with significantly increased COPD risk (adjusted odds ratio [OR] = 1.58 with 95% confidence interval [CI] = 1.14–2.15, P = 0.0056 for rs6435156C>T; adjusted OR = 1.47 and 95% CI = 1.10–1.97, P = 0.0092 for rs1048829G>T). Older age, cigarette smoking, family history of cancer and COPD were all factors that interacted with rs6435156C>T and rs1048829G>T causing increased COPD risk. Cigarette smokers with rs6435156 (CT + TT) or rs1048829 (GT + TT) were more susceptible to COPD than that with the rs6435156CC or rs1048829GG genotypes. In A549 human alveolar epithelial cells, luciferase reporter assays revealed that introduction of 3′UTR of *BMPR2* plasmids carrying rs6435156T allele but not rs1048829T led to lower luciferase activity than the wild-type C or G alleles. Comparing to rs6435156CC, treatment with hsa-miR-20a mimics deceased whereas hsa-miR-20a inhibitor restored the luciferase reporter activity in cells transfected with constructs carrying rs6435156TT. BMPR2 mRNA and protein expressions were significantly lower in PBMCs from COPD smokers than that in non-smokers. COPD patients carrying rs6435156T allele had less BMPR2 expression in PBMCs.
Interpretation: This study demonstrated that both rs6435156C>T and rs1048829G>T variants in *BMPR2* contributed to increased susceptibility to COPD. The T variants of rs6435156 increased COPD risk likely by binding with hsa-miR-20a, thus leading to downregulated BMPR2 expression in lung epithelial and immune cells.

1. Introduction

Chronic obstructive pulmonary disease (COPD), characterized by progressive and irreversible airflow limitation, is a growing public health burden and might become the third leading cause of death worldwide by 2020 (Raherison and Girodet, 2009). Various risk factors, including environmental factors (mainly cigarette smoking), infection, and genetic susceptibility are involved in the occurrence of COPD. The lung pathology of this disease is featured by abnormal inflammation, small airway wall structural remodeling, mucus hypersecretion and/or emphysema (Rabe et al., 2007; Arinir et al., 2009). Imbalanced inflammation and anti-inflammation, proteases and antiproteases, and oxidation and antioxidation are thought to be the primary pathological mechanisms underlying COPD, and abnormal inflammation has been suggested to play the central role. Therefore, identifying the genetic

determinants to inflammation will eventually benefit the early diagnosis and prevention of this disease.

Bone morphogenetic protein receptor type 2 (BMPR2), a member of the transforming growth factor β receptor superfamily of the transmembrane serine/threonine kinase receptors, is expressed at high level in various tissues including pulmonary vasculature and airway epithelium (Favre et al., 2003; Atkinson, 2002). Signaling through BMPR2 is essential for embryonic development, pulmonary vascular cell growth and differentiation, angiogenesis, organogenesis, endothelial cell and smooth muscle cell interaction (Shiraishi, 2012; Teichert-Kuliszewska et al., 2006; Yang et al., 2005). Bone morphogenetic protein (BMP) signaling pathway is not only involved in maintaining normal vascular morphology, but also in regulating pro-inflammatory responses in vasculature (Teichert-Kuliszewska et al., 2006; Nohe et al., 2002). A recent report by Kim et al. found that BMPR2 had unique anti-inflammatory functions among BMP receptors (Kim et al., 2013). Another study demonstrated that BMPR2 expression was decreased in lung tissue samples from healthy smokers and COPD patients (Llinàs et al., 2011). Yet, the exact role of BMPR2 on COPD development and progression are unknown.

The human *BMPR2 gene* is located at chromosome 2q33, encoding a 12,086 bp messenger RNA (mRNA) and 13 exons with 1038 amino acids. *BMPR2* is highly polymorphic with 3317 identified single-nucleotide polymorphisms (SNPs) (Fig. 1A). Although *BMPR2* mutations are well defined to be responsible for the occurrence of majority of heritable pulmonary arterial hypertension (PAH) and idiopathic PAH cases (Rosenzweig et al., 2008; Sztrymf et al., 2008; Guo et al., 2011; Wang et al., 2013; Yang et al., 2012), their relationship to COPD has not been identified. The 3′-untranslated region (3′UTR) of gene generally plays important roles in regulating mRNA stability, thus affecting the translation efficacy and protein expression by interacting with other post-transcriptional regulatory factors such as microRNAs (miRNAs). SNPs in 3′UTR could modulate the function of 3′UTR if they are located in the binding sites for miRNAs. In this study, we hypothesized that the presence of SNPs in 3′UTR of *BMPR2* may regulate BMPR2 expression, and therefore contribute to COPD risk by interacting with environmental factors such as cigarette smoking. Therefore, we selected two SNPs in the 3′UTR of *BMPR2*, rs6435156C>T and rs1048829G>T, genotyped them in a southern Chinese population, and assessed their functional association with miRNA, level of BMPR2 expression and risk of COPD.

2. Materials and Methods

2.1. Study Subjects

This study was conducted in 594 COPD patients and 600 age (±5 years) and sex frequency matched healthy control subjects of southern Chinese origin. All subjects were enrolled from the First Affiliated Hospital of Guangzhou Medical University (Guangzhou, Guangdong, China) from January 2010 to December 2012. The COPD diagnosis was according to the Global Initiative for Obstructive Lung Disease (GOLD) guidelines (Rabe et al., 2007). The inclusion criteria for COPD included the following: 1) presence of chronic airway symptoms and signs, such as chronic cough, dyspnea, sputum production, and wheezing, etc.; 2) indication of chronic airway obstruction which was defined as a forced expiratory volume in 1 (FEV1) /forced vital capacity (FVC) < 70% after inhalation of 400 μg salbutamol. All the participants were ethnic Han Chinese and they shared no kinship with each other. All controls were excluded if they had a history of chronic respiratory disease, atopy, an acute pulmonary infection during the 4 weeks before assessment for participation in this study, a family history of COPD, blood transfusion in the last 6 months, or evidence of airflow obstruction ($FEV_1/FVC < 70\%$). All participants were asked to provide data on smoking status, and to donate 5 ml of peripheral blood after an informed consent was obtained in written form. The project was approved by the institutional review boards of Guangzhou
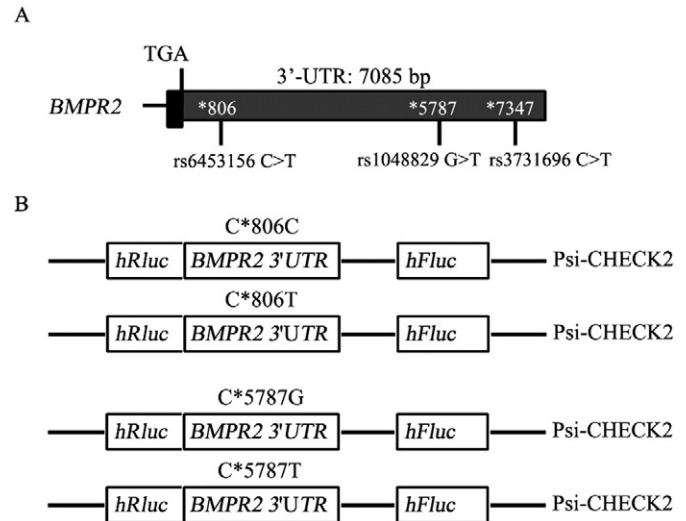

Fig. 1. Genomic structure of *BMPR2* and reporter gene constructs for the 3′UTR of *BMPR2*. (A) Location of the three validated SNPs (rs6435156C>T, rs1048829G>T and rs3731696C>T) in 3′UTR of *BMPR2* gene. (B) Schematic drawing of the reporter gene constructs containing a 618 bp of 3′UTR of *BMPR2* with rs6435156C or T allele, or a 620 bp of 3′UTR of *BMPR2* with rs1048829G or T allele.

Medical University (Ethics Committee of The First Affiliated Hospital (GZMC2009-08-1336).

2.2. SNP Selection

SNPs for CHB were obtained from both dbSNP and HapMap databases and analyzed using Haploview software 4.2. In this study, we originally selected three SNPs, rs6435156, rs1048829 and rs3731696, located at the 3′UTR of *BMPR2* according to the following two criteria: (1) The minor allele frequency (MAF) > 0.05; (2) r^2 threshold of 0.8, as analyzed by a pair-wise tagging algorithm. Next, we performed linkage disequilibrium (LD) analysis in controls and found that rs6435156 was in incomplete LD with rs1048829 ($D' = 1$ and $r^2 = 0.704$); in complete LD with rs3731696 ($D' = 1$ and $r^2 = 0.98$), rs1048829 and rs3731696 were in incomplete LD with each other ($D' = 0.98$ and $r^2 = 0.69$). Therefore, we chose rs6435156 and rs1048829 to represent all three of them for subsequent genotyping and functional analyses.

2.3. Plasmid Construction

The rs6435156C allele reporter construct was prepared by amplifying a 618 bp 3′UTR of human *BMPR2* by using the forward primer: 5′-CCG CTC GAG TCA CAT TGT CAA ACA GAA TTT TTC-3′ and reverse primer: 5′-ATT TGC GGC CGC AAA GTC ACC AGT CTT TGC TTG G-3′. The rs1048829G allele reporter construct was prepared by amplifying a 620 bp 3′-UTR of human *BMPR2* by using the forward primer: 5′-CCG CTC GAG ATC GAG AGT TAA GAT GTT TCT ATT TGA-3′ and reverse primer: 5′-ATT TGC GGC CGC TGG GTT TCA AGT TGT TTT AAA AAT G-3′. The PCR products were ligated into Psi-CHECK2 basic vector in downstream of renilla luciferase gene at the XhoI and NotI restriction sites (Promega, Madison, WI) to produce a *BMPR2*-Psi-CHECK2 reporter plasmid. The subsequent mutation of T for rs6435156C or rs1048229G in these constructs was generated with the MutanBEST KIT (TaKaRa, Dalian, China) according to the manufacturer's protocol. All constructs were sequenced to ensure correct sequence, orientation and integrity of each insert (Fig. 1B). The function of the cloned fragments on upstream

gene expression was determined by renilla luciferase activity with fire-fly luciferase as internal standard.

2.4. Statistical Analysis

All statistical analyses were run with SAS software (version 9.2; SAS Institute, Cary, NC, USA), and the two-tailed $P < 0.05$ was considered statistically significant. χ^2 test was used to evaluate differences in the distributions of demographic characteristics, selected variables, and genotypes of the variants. Goodness of fit to the Hardy–Weinberg equilibrium expectation in control was also evaluated by the χ^2-test for each SNP. Akaike's information criteria (AIC) were applied to select the most parsimonious genetic model for each SNP (Kamikubo et al., 1986). Odds ratios (ORs) and its corresponding 95% confidence intervals (CIs) were measured by an unconditional logistic regression model with adjustment for age, sex, smoking status, and family history of cancer. Stratification analyses were also conducted by variables of interest such as age, sex, smoking status, and family history of cancer etc. The pairwise LD among the SNPs was calculated with Lewontin's standardized coefficient D′ in the publicly available Haploview 4.2 software (http://www.broad.mit.edu/personal/jcbarret/haplo/) with default settings (the CI for a strong LD was minimal for upper 0.98 and low 0.7 and maximal for a strong recombination of 0.9, and a fraction of strong LD in informative comparisons was at least 0.95). LD coefficiency r^2 and haplotype blocks were defined by the method of Gabriel et al. (2002). PHASE 2.1 Bayesian algorithm was used to validate the haplotype frequencies estimated by Haplo.stats (Stephens and Donnelly, 2003). In case of multiple comparisons, the P values was adjusted in a logistic regression model that included age, sex, smoking status, family history of cancer, and corrected by Bonferroni test for each of the two SNPs, where $P < 0.025$ was considered to be significant. For the in vitro study, the differences between two or among multiple treatment groups were analyzed by Student's t-test or ANOVA, respectively.

2.5. Other Methods

The procedures of genomic DNA extraction and genotyping, real-time PCR, western blotting, RNA interferences, luciferase reporter assay, CSE preparation and cell treatment were described in the supplemental Materials and Methods.

3. Results

3.1. Clinical and Demographical Characteristics of the Study Population

The selected clinical and demographical characteristics of the recruited subjects are summarized in Table 1. There was no significant deviation in distribution of age, sex, and family history of cancer between the two cohorts of case and control subjects. Significant differences in smoking status, pack-year, passive smoking, family history of COPD, FEV1% predicted, and FEV1/FVC% were observed between the case subjects and controls ($P < 0.0001$). There were more male smokers in cases than in controls ($P < 0.0001$).

3.2. Association of rs6435156C>T and rs1048829G>T With COPD Risk

The genotype frequencies of the two tag SNPs (rs6435156 and rs1048829) in the 3′UTR of BMPR2 in the study population fit the HWE ($P > 0.05$ for all). Multivariate logistic regression models demonstrate that after adjusting for confounding factors (such as age, sex, smoking status and family history of cancer), significant associations were observed for the two tag SNPs (adjusted $P = 0.0028$ for rs6435156 and $P = 0.0030$ for rs1048829 in an additive model) based on the best fit of AIC; compared with wild-type carriers in a dominant model, a significantly increased COPD risk was associated with the variant genotypes of rs6435156C>T (adjusted OR = 1.57 and 95% CI =

Table 1
Frequency distributions of selected clinical and demographic characteristics of COPD patients and controls.

Variables	Cases	Controls	P^a
	n = 594	n = 600	
	n (%)	n (%)	
Age (years)			
≤60	249 (41.91)	336 (56.00)	**0.0006**
>60	344 (58.01)	264 (44.00)	
Sex			
Male	512 (86.78)	495 (82.89)	0.1864
Female	78 (13.22)	102 (17.11)	
Smoking status			
Current	74 (12.54)	178 (29.82)	**<0.0001**
Former	418 (70.85)	6 (1.01)	
Never[b]	98 (16.61)	413 (69.18)	
Packs year			
0	98 (16.59)	414 (69.46)	**<0.0001**
0–20	80 (13.54)	122 (20.47)	
20–	413 (69.88)	60 (10.07)	
Passive smoke			
Yes	459 (77.66)	100 (16.78)	**<0.0001**
No	132 (22.34)	496 (83.22)	
Sex and smoking			
Male smokers	473 (80.00)	178 (29.87)	**<0.0001**
Male nonsmokers	40 (6.78)	317 (53.02)	
Female smokers	20 (3.38)	6 (1.01)	
Female nonsmokers	58 (9.81)	96 (16.11)	
Family history of cancer			
Yes	8 (1.36)	6 (1.01)	0.6938
No	583 (98.64)	591 (98.99)	
Family history of COPD			
Yes	70 (11.86)	6 (1.01)	**<0.0001**
No	521 (88.14)	591 (98.99)	
GOLD			
I	88 (14.89)		
II	94 (15.91)		
III	223 (37.73)		
IV	186 (31.47)		
Age	60.22 ± 9.91	60.61 ± 7.50	0.5974
FEV1%, predicted	42.24 ± 20.48	94.26 ± 15.69	**<0.0001**
FEV1/FVC, %	46.93 ± 17.67	81.41 ± 6.85	**<0.0001**

[a] P values for a two-sided χ^2 test or t-test; in bold are P values <0.05.
[b] Individuals who had smoked fewer than 100 cigarettes in their lifetime.

1.14–2.15 for CT/TT genotypes, $P = 0.0056$) and rs1048829G>T (adjusted OR = 1.47 and 95% CI = 1.10–1.97 for GT/TT, $P = 0.0092$) (Table 2).

3.3. Stratification Analysis of rs6435156C>T and rs1048829G>T

Next, we next assessed the associations of the rs6435156C>T and rs1048829G>T variant genotypes with COPD risk stratified by selected variables listed in Table 1. As seen in Table 3, compared with the common wild-type homozygous genotype CC of rs6435156, the adverse effect of CT plus TT genotypes on COPD risk was significant in the older group (adjusted OR = 2.00 with 95% CI = 1.27–3.15), never-smokers (adjusted OR = 2.47 with 95% CI = 1.50–4.09), females (adjusted OR = 3.59 with 95% CI = 1.73–7.44), 0 pack-years of cigarette smoke (adjusted OR = 2.48 with 95% CI = 1.50–4.10), no family history of cancer (adjusted OR = 1.54 with 95% CI = 1.21–2.11), no passive smoking subjects (adjusted OR = 2.08 with 95% CI = 1.28–3.37), without family history of COPD (adjusted OR = 1.48 with 95% CI = 1.06–2.04), and in those with more severe stage (GOLD IV) of COPD (adjusted OR = 1.83 with 95% CI = 1.19–2.83). Drastic interactions on COPD risk were observed between rs6435156T variants and age (adjusted $P = 4.0 \times 10^{-4}$), smoking status (adjusted $P = 3.8 \times 10^{-11}$), pack year of cigarette smoking (adjusted $P = 1.7 \times 10^{-14}$), and passive smoking ($P = 6.0 \times 10^{-9}$) (Table 3). Analyses of rs1048829T variants revealed similar results to rs6435156C>T (Table 3).

Table 2
Distribution of rs1048829G>T and rs6435156C>T genotypes and their associations with risk of COPD.

Genotypes/alleles	Case	Control	Crude	Adjusted[a]		
	(N = 594)	(N = 600)	OR (95% CI)	OR (95% CI)	P	AIC
	n (%)	n (%)				
No. of subjects	594	600				
No. of alleles	1188	1200				
rs1048829G>T						
Co-dominant model						
GG	368 (62.37)	423 (70.81)	1.00 (ref.)	1.00 (ref.)		1252
GT	192 (32.54)	158 (26.51)	1.39 (1.08–1.79)	1.37 (1.01–1.86)	0.0403	
TT	30 (5.08)	16 (2.68)	2.15 (1.54–4.01)	**2.58 (1.21–5.51)**	**0.0140**	
Additive model			1.42 (1.16–1.75)	**1.46 (1.14–1.87)**	**0.0030**	1249
Dominant model						
GT + TT	222 (37.63)	174 (29.19)	1.46 (1.15–1.87)	**1.47 (1.10–1.97)**	**0.0092**	1251
T allele	0.21	0.16				
rs6435156C>T						
Co-dominant model						
CC	417 (70.27)	476 (79.33)	1.00 (ref.)	1.00 (ref.)		1255
CT	158 (26.69)	118 (19.67)	1.53 (1.17–2.01)	**1.49 (1.07–2.06)**	**0.0178**	
TT	18 (3.04)	6 (1.00)	3.43 (1.35–8.73)	**3.32 (1.21–9.13)**	**0.0202**	
Additive model			1.61 (1.27–2.04)	**1.55 (1.16–2.06)**	**0.0028**	1253
Dominant model						
CT + TT	176 (29.73)	124 (20.67)	1.62 (1.25–2.12)	**1.57 (1.14–2.15)**	**0.0056**	1254
T allele	0.16	0.11				

P$_{HWE}$: calculated by the Hardy–Weinberg equilibrium among the control subjects (HWE = 0.58 for rs1048829, HWE = 0.93 for rs6435156).
Power (1-β): for rs1048829 is 0.95 in additive model and 0.89 in dominant model; for rs6435156 is 0.99 in additive model and 0.96 in dominant model.
[a] Adjusted in a logistic regression model that included age, sex, smoking status, and family history of cancer, and corrected by Bonferroni test at a significance level of 0.05/2 = 0.025 for each of the two tested SNPs; in bold are *P* values <0.025.

3.4. Effects of Haplotypes and Combined Genotypes of rs6435156C>T and rs1048829G>T on COPD Risk

The haplotypes and combined genotypes analyses were both performed to determine the separate and joint effects of rs6435156C>T and rs1048829G>T on COPD risk (Table 4). Compared to the major common haplotype G-C, the haplotype T–T of rs6435156 and rs1048829 showed significant association with increased COPD risk (adjusted OR = 1.25, 95% CI = 1.08–1.44 and P = 0.002) (Table 4). In combined genotypes risk analysis, we found that the presence of

Table 3
Stratification analysis of rs1048829G>T and rs6435156C>T on COPD risk.

Variables	rs1048829G>T				rs6435156C>T			
	Case[a]	Control[a]	OR (95%CI)[b]	Adjusted P[c]	Case[a]	Control[a]	OR (95%CI)[b]	Adjusted P[c]
	GG\GT + TT	GG\GT + TT			CC\CT + TT	CC\CT + TT		
Age				**7.9 × 10⁻³**				**4.0 × 10⁻⁴**
≤60	156/92	240/94	1.48 (0.97–2.26)		175/75	264/70	1.28 (0.82–2.02)	
>60	212/130	183/80	1.45 (0.97–2.17)		242/101	212/54	**2.00 (1.27–3.15)**	
Sex				0.05				**0.01**
Male	324/188	348/146	1.27 (0.91–1.76)		366/148	392/104	1.25 (0.88–1.79)	
Female	88/34	75/28	**2.57 (1.32–5.01)**		100/56	84/20	**3.59 (1.73–7.44)**	
Smoking status				**3.7 × 10⁻¹⁸**				**3.8 × 10⁻¹¹**
Yes	314/178	126/58	1.21 (0.84–1.74)		356/138	138/44	1.12 (0.76–1.66)	
No	54/44	297/116	**1.96 (1.22–3.16)**		60/38	338/78	**2.47 (1.50–4.09)**	
Pack year				**1.4 × 10⁻²¹**				**1.7 × 10⁻¹⁴**
20–	260/152	32/28	0.66 (0.38–1.14)		299/115	40/20	0.74 (0.41–1.32)	
0–20	54/26	92/30	1.57 (0.83–3.00)		58/23	98/24	1.39 (0.70–2.73)	
0	54/44	299/116	**1.97 (1.22–3.18)**		60/38	340/78	**2.48 (1.50–4.10)**	
Passive smoke				**1.6 × 10⁻¹⁴**				**6.0 × 10⁻⁹**
Yes	284/174	64/36	1.16 (0.72–1.88)		326/134	72/28	1.02 (0.61–1.69)	
No	84/48	359/138	1.55 (0.98–2.43)		80/42	404/94	**2.08 (1.28–3.37)**	
Family history of cancer				0.98				0.98
Yes	4/4	6/2	1.11 (0.87–1.37)		4/4	10/4	1.17 (0.93–1.52)	
No	364/218	417/174	**1.45 (1.08–1.94)**		413/172	466/120	**1.54 (1.12–2.11)**	
Family history of COPD				0.97				0.97
Yes	40/30	8/4	1.04 (0.79–1.26)		22/13	41,279	1.06 (0.87–1.18)	
No	328/192	415/170	**1.42 (1.05–1.92)**		186/75	234/60	**1.48 (1.06–2.04)**	
GOLD				—				—
I	54/34	423/174	1.53 (0.94–2.51)		60/28	476/121	1.74 (0.89–3.12)	
II	60/34	423/174	1.33 (0.80–2.23)		68/26	476/121	1.36 (0.78–2.38)	
III	156/66	423/174	0.99 (0.67–1.46)		168/54	476/121	1.08 (0.71–1.64)	
IV	98/88	423/174	**2.15 (1.43–3.25)**		120/68	476/123	**1.83 (1.19–2.83)**	

[a] Dominant model: Wild homozygote \ Heterozygote + Variant homozygote.
[b] Adjusted in a logistic regression model that included age, sex, smoking status, and family history of cancer, and corrected by Bonferroni test at a significance level of 0.05/2 = 0.025; in bold are *P* values <0.025.
[c] *P* value: calculated by gene–environment interaction.

individual risk genotype of rs6435156T or rs1048829T (defined as "1") did not cause significant influence on COPD risk; however, compared to "0" variants, there was a significantly increased risk of COPD as the number of risk genotypes T increased to "2" (adjusted OR = 1.26, 95% CI = 1.10–1.48 and $P = 0.005$), indicating that these variants had a joint effect on the risk of COPD (adjusted trend test $P = 0.0012$) (Table 4). Nevertheless, compared with rs1048829G>T ($P = 0.926$), rs6435156C>T took the main effect ($P = 0.043$) on COPD risk, as revealed by regression analysis when two of them were included in the same regression model.

3.5. Effects of rs6435156 and rs1048829 Polymorphisms on BMPR2 Gene Reporter Activity

To determine the effects of the rs6435156C>T and rs1048829G>T polymorphisms on *BMPR2* expression, four constructs with a fragment of *BMPR2* 3′UTR carrying rs6435156C or T, rs1048829G or T ligated in downstream of a luciferase reporter gene were generated and transfected into A549 cells. As shown in Fig. 2A, the transcription activity of the reporter gene in cells carrying rs6435156T allele was drastically lower than that those carrying C allele ($P = 6.7 \times 10^{-6}$ for rs6435156T versus C), suggesting that the T variant of rs6435156 negatively modulated BMPR2 expression in A549 cells. However, the luciferase reporter activity was not different between cells transfected with vector carrying rs1048829T and G allele (Fig. 2B).

3.6. Effects of rs6435156C>T Genotypes on BMPR2 Expression in PBMCs From COPD Subjects

In order to determine the influence of rs6435156C>T polymorphism on *BMPR2* expression in COPD patients, we assessed the levels of *BMPR2* mRNA and protein in PBMCs from COPD patients carrying CC, CT or TT genotype of rs6435156. The CT carriers exhibited significant reduced mRNA and protein expression of *BMPR2* comparing with the CC carriers. The level of *BMPR2* mRNA and protein expression was further reduced in PBMCs from COPD subjects with rs6435156TT genotype compared to CT (Fig. 3).

3.7. Synergistic Effects of rs6435156T Variants and Cigarette Smoking on BMPR2 Expression

Considering that cigarette smoking is a pivotal risk factor in COPD pathogenesis, we investigated the rs6435156 polymorphism distribution in smoking and non-smoking COPD patients. As shown in Fig. 4A–C, cigarette smoking induced greater decreases of *BMPR2* mRNA and protein level in PBMCs from COPD smokers than from non-smokers, with further reduction in those smoking patients carrying rs6435156CT genotype. Due to limited sample size available, we didn't
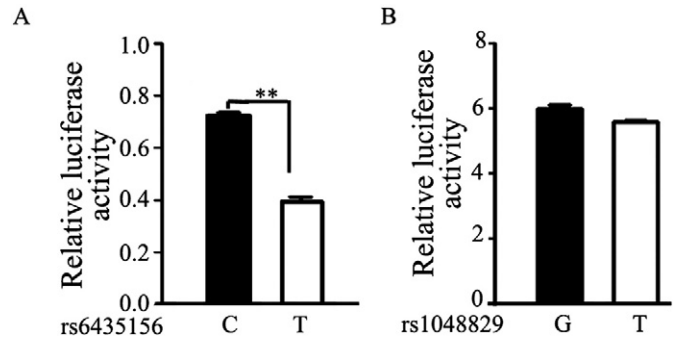

Fig. 2. Luciferase activity of the constructed plasmids in A549 cells. Constructs containing rs6435156C or T allele (A) or rs1048829G or T allele (B) were transfected into A549 cells and the luciferase activity was measured. The renilla luciferase activity of each construct was normalized against the activity of firefly luciferase. Data were from three triplicate experiments and shown as mean ± SD. (**$P < 0.01$).

evaluate the association of *BMPR2* expression level and genotype rs6435156TT under cigarette smoking. According to these results, we hypothesize that rs6435156C>T polymorphism is associated with *BMPR2* expression reduction in COPD patients, especially in those with cigarette smoking history.

To further verify the association between rs6435156C>T and cigarette smoking on modulating *BMPR2* expression, A549 cells were transfected with the luciferase reporter construct carrying rs6435156C or T genotype and then treated with 5% CSE for 72 h. CSE significantly reduced the luciferase activity in cells carrying rs6435156C or T constructs; however, the more obvious reduction of luciferase activity was observed in cells carrying T construct ($P = 6.7 \times 10^{-5}$) (Fig. 4D). This result suggests that rs6435156T variant potentiated cigarette smoking induced *BMPR2* downregulation in pulmonary epithelial cells.

3.8. Effects of miR-20a on BMPR2 Expression

The 3′UTR of genes generally functions by binding with microRNA and therefore modulate mRNA stability and gene expression. We subjected the sequence of rs6435156 to a bioinformatics analysis (http://snpinfo.nih.gov/) and found that rs6435156 located at the potential binding site of has-miR-20a (Fig. 5A). The reporter constructs rs6435156CC or TT was transfected into A549 cells with co-transfection of miR-20a mimics or inhibitor. When compared with cells without co-transfection, the mimics of miR-20a could suppress the mRNA expression of *BMPR2* gene in A549 cells with rs643515TT genotype. However, the reduction of luciferase activity could be recovered by co-transfected hsa-miR-20a inhibitors in both cells

Table 4
Effect of haplotypes and combined genotypes of rs1048829G>T and rs6435156C>T on COPD risk.

Genotypes	Cases	Controls	Crude	Adjusted	
	n (%)	n (%)	OR (95%CI)	OR (95%CI)[b]	P[a]
No. of subjects	594	600			
No. of haplotypes	1188	1200			
Haplotypes[b]					
G-C	928 (48.08)	1001 (51.92)	1.00 (ref.)	1.00 (ref.)	
T-C	58 (49.15)	60 (50.85)	1.04 (0.72–1.51)	1.24 (0.79–1.97)	0.352
T–T	194 (59.88)	130 (40.12)	1.27 (1.13–1.43)	**1.25 (1.08–1.44)**	**0.002**
Risk genotypes[c]					
0	368 (46.58)	421 (53.42)	1.00 (ref.)	1.00 (ref.)	
1	46 (47.92)	50 (52.08)	1.06 (0.69–1.61)	1.16 (0.69–1.97)	0.577
2	175 (58.67)	124 (41.33)	1.28 (1.12–1.46)	**1.26 (1.10–1.48)**	**0.005**
Trend test P value			**0.0006**	**0.0012**	

[a] Adjusted in a logistic regression model that included age, sex, smoking status, and family history of cancer.
[b] Haplotype constructed with order of SNPs: rs1048829G>T and rs6435156C>T of BMPR2; if frequency of haplotype is below 0.05, then delete such as G-T.
[c] Genotype combinations in *BMPR2*: rs1048829T variant genotypes GT and TT and rs6435156T variant genotypes CT and TT are defined as risk genotypes.
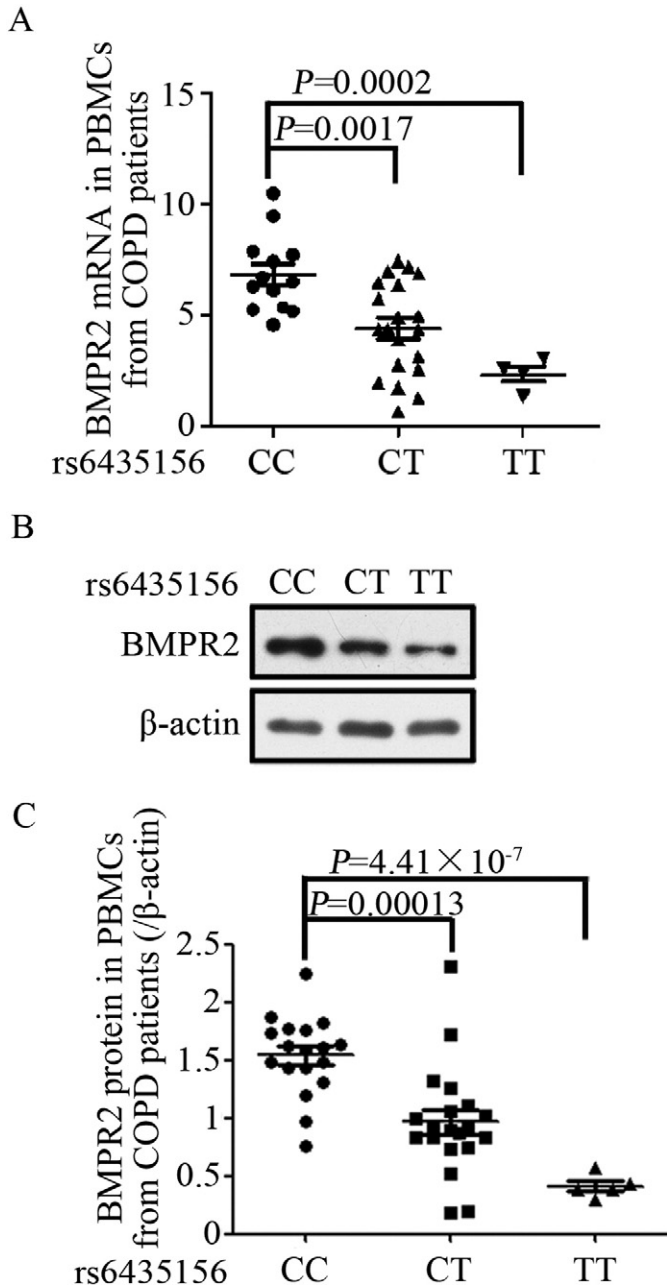
A

B

C

Fig. 3. BMPR2 expression levels in PBMCs from COPD patients carrying SNP rs6435156C>T. (A) *BMPR2* mRNA levels were determined by quantitative RT-PCR with 18s rRNA as internal control. (Individual rs6435156C>T genotype: CC, n = 18; CT, n = 20; TT, n = 4). (B) *BMPR2* protein levels in PBMCs carrying rs6435156C>T genotype were detected by western blotting. The level of β-actin was taken as internal control. Data were randomly selected from 44 individuals detected in one experiment (CC, n = 18; CT, n = 20; TT, n = 4). (C) Relative protein expression level by rs6435156C>T genotype was calculated in 44 individuals via band intensity quantification and normalization to internal control β-actin. Data were shown as mean ± SD and analyzed with Student's t-test.

transfected with rs6435156CC and TT (Fig. 5B). These results suggested miR-20a target to rs6435156 in 3′UTR of *BMPR2* mRNA and lead to decreased BMPR2 expression. The rs6435156T variant potentiates the binding of miR-20a to this site, which results in greater downregulation of BMPR2.

4. Discussion

The present study demonstrated that both rs6435156C>T and rs1048829G>T variant genotypes in the 3′UTR of *BMPR2* were

associated with increased risk of COPD in a southern Chinese population. COPD smokers carrying the T variant (CT and TT) of rs6435156 exhibited significantly reduced mRNA and protein expression of BMPR2 in PBMCs comparing with the CC carrier smokers. Moreover, we found that the rs6435156T variants significantly decreased the expression of BMPR2 both alone and in synergy with cigarette smoke exposure in lung epithelial cells. The miRNA hsa-miR-20a was responsible for decreased expression of BMPR2 in cells carrying rs6435156T genotype.

Reduction of BMPR2 expression was observed in the lung tissue of COPD patients (Llinàs et al., 2011). However, none has been known about the association between *BMPR2* mutation and COPD. As an identified risk factor, *BMPR2* gene mutations have been shown to be related with the occurrence of heritable and idiopathic PAH. Deficiency of *BMPR2* induced more obvious perivascular inflammation and muscularization characterized by increased thickness of small pulmonary arterioles in *BMPR2* mutants than in wild type mice with pulmonary hypertension (PH) (Song et al., 2008). Though PH is a common comorbidity of COPD, *BMPR2* gene mutation has not been assessed as a risk factor for COPD. This may be due to the complex nature of COPD pathology, which has been recognized to involve interactions of multiple genes and environment factors.

In this study, we identified that both rs6435156C>T and rs1048829G>T in 3′UTR of *BMPR2* were significantly associated with increased COPD risk, when analyzed separately and in a joint manner. However, it was rs6435156T, but not rs1048829T variant found being associated with decreased BMPR2 expression in lung epithelial cells when assessed by luciferase reporter assay. The fact that both rs6435156T and rs1048829T variants were significant when included in the same regression model further suggests that the association of rs1048829 with COPD is not due to the LD with rs6435156. The apparent discrepancy between the above population study and in vitro functional study is likely due to the effects of rs1048829G>T on COPD risk is not strong enough to be monitored in our tested in vitro system. Consistently, we found rs6435156C>T took the main effect on COPD risk comparing to rs1048829G>T when regression analysis was performed with two of the SNPs included in the same regression model. In addition, it is also likely that rs1048829G>T functions by affecting rs6435156C>T, thus presenting a joint effect on COPD risk; yet, this assumption needs to be further investigated in the future study.

In this study, we showed that rs6435156T variant genotype is associated with reduced *BMPR2* expression in PBMCs from COPD non-smokers and with further reduction in COPD smokers. Moreover, consistent with these results from the in vivo study, we found that, although CSE decreased the luciferase reporter activity in cells carrying rs6435156C, it caused greater decrease of the reporter activity in cells carrying rs6435156T variant (Fig. 4D). According to bioinformatics analysis, we found the rs6435156C>T resides in the potential binding site of has-miR-20a. MicroRNA miR-20a was shown to regulate BMP signaling pathway through targeting on the binding sites of 3′UTR of BMP-2 and possibly *BMPR2* gene in fish skeletogenesis (Tiago et al., 2014). Treatment of antagomiR-20a restored the functional levels of *BMPR2* expression in pulmonary arteries (Brock et al., 2014). Interestingly, we found treatment of miR-20a mimics increased, and miR-20a inhibitor decreased the luciferase activity in cells carrying rs6435156TT variants. In contrast, both miR-20a mimics and inhibitor did not change the level of luciferase activity in cells carrying rs6435156CC genotypes. These results indicate that cigarette smoke normally could downregulate BMPR2 expression independent of has-miR-20a; however, under the presence of rs6435156T variant, it further decreases BMPR2 expression in has-miR-20a dependent manner; the rs6435156C>T mutation is responsible for downregulated BMPR2 expression likely by recruiting the binding of miR-20a to the 3′UTR of *BMPR2*. Consistently, Brock M. et al. identified that expression of *BMPR2* was modulated by a STAT3-miR-17/92-*BMPR2* pathway, in which miRNA cluster 17/92 (miR-17/92) encodes hsa-miR-20a. A highly conserved STAT3-binding

A

B

C

D

A

Position 788-809 of *BMPR2* 3'UTR   5' ... ... ATGUUUCCUUCAAGGCAU(U/C)UUA..

rs6435156   hsa-miR-20a   3' GAUGGACGUGAUAUUCGUG A A A
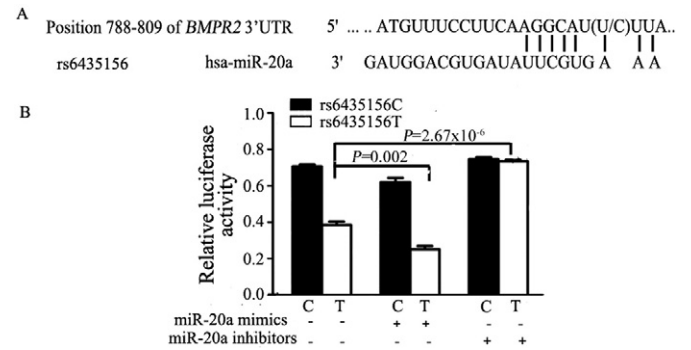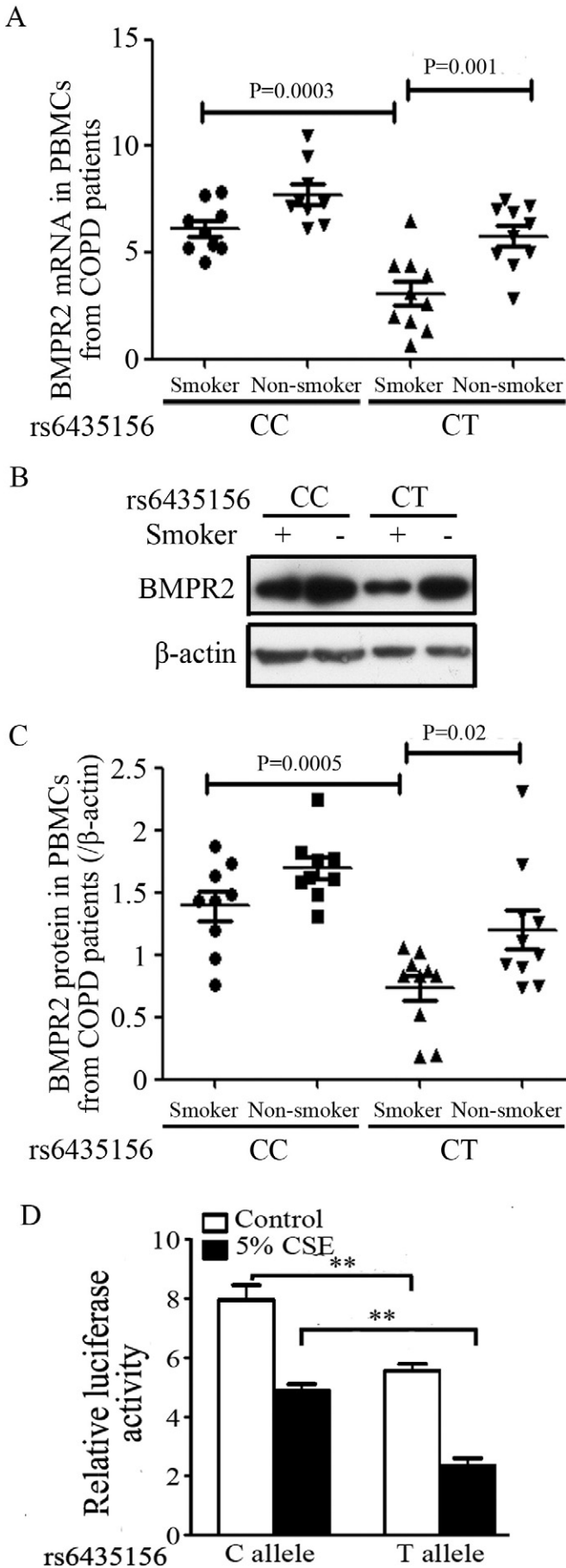
B

Fig. 5. Association of has-miR-20a and *BMPR2* gene expression. (A) Proposed binding of has-miR-20a to 3′UTR of *BMPR2*. The SNP rs6435156C>T is located within the seed region of the binding site with the T allele perfectly matching the corresponding C allele in miR-20a. (B) Luciferase activity in A549 cells carrying rs6435156C or T allele under co-transfection of miR-20a mimics or miR-20a inhibitors. Data were mean of three triplicate experiments and shown as Mean ± SD.

site was found in the promoter region of the miR-17/92 gene and mediated IL-6 modulated *BMPR2* expression (Brock et al., 2009). As a proinflammatory factor, IL-6 was upregulated by cigarette smoking in lung tissue from COPD patients (Llinas et al., 2011). Plasma IL-6 level was suggested to be a persistent and progressive parameter in evaluating systemic inflammatory process and mortality association in COPD patients (Ferrari et al., 2013). Based on the above evidence from us and others, we propose that the higher risk of COPD in cigarette smoking populations carrying rs6435156T variant is likely due to IL-6 and hsa-miR-20a mediated downregulation of BMPR2 expression.

Given that A549 cells we used are not derived from the small airways but are a transformed cell line with a pneumocyte phenotype, and our BMPR2 protein and mRNA expression work was performed in PBMCs but not lung tissue, we performed eQTL look-up in lung cell types (including whole human lung) to see whether or not the studied SNPs rs6435156C>T and rs1048829G>T indeed act as cis acting eQTLs for BMPR2. Based on calculation in the online free resources of SNPexp and GENE Expression VARiation (Genevar), only rs1048829 was found to act as cis acting eQTLs for BMPR2 though with weak statistical difference ($P = 0.05$). The possible reason for the conflicting results between eQTL results and the functional results we obtained in this study might be due to the bank data in SNPexp was not derived from Chinese population and Genevar was not from COPD patients (Cheung et al., 2012; Holm et al., 2010; Yang et al., 2010).

It is generally believed that the initial event in the natural history of PH associated with COPD could be due to endothelial dysfunction and inflammation caused by risk factors including cigarette smoke (Barbera and Blanco, 2009). Although the actual role of BMPR2 in PH or COPD development are not fully understood, it was known playing an anti-inflammatory function in endothelial cells in response to proatherogenic stimuli (Kim et al., 2013). In *BMPR2* heterozygous mutant mice, BMPR2 deficiency leads to enhanced proinflammatory cytokine production in pulmonary artery smooth muscle cells (Davies et al., 2012). BMP4, a ligand of BMPR2, participates in controlling

Fig. 4. Effects of SNP rs6435156C>T on BMPR2 expression under cigarette smoke exposure. The mRNA and protein level of *BMPR2* were determined in PBMCs from 38 COPD patients with rs6435156C>T genotype (CC, non-smoker = 9, smoker = 9; CT, non-smoker = 10, smoker = 10). (A) Relative mRNA level was determined by quantitative RT-PCR. (B) The *BMPR2* protein levels with β-actin as internal control. Data were randomly selected from the 38 individuals. (C) Relative protein expression level by rs6435156C>T genotype was calculated in 38 individuals via band intensity quantification and normalization to internal control β-actin. Data were shown as mean ± SD and analyzed with Student's t-test. (D) A549 cells were transfected with luciferase reporter constructs containing rs6435156C or T allele and subjected to culture with cigarette smoke extract (CSE, 5%). The relative luciferase activity was measured. Data were from three triplicate experiments and shown as mean ± SD. (**$P < 0.01$).

LPS-induced inflammatory responses in lung epithelial cells (Li et al., 2014). Collectively, these evidence suggest that BMPR2 could be a critical anti-inflammatory factor in the lung, where the mutation of BMPR2 contributes to uncontrolled inflammation and development of COPD. Considering both genetic and environmental factors were involved in COPD pathology, we further tested the interaction between *BMPR2* mutation and cigarette smoking on COPD risk. Our results from both population-based in vivo and cell-based in vitro study confirmed that the rs6435156C>T mutation in 3′UTR of *BMPR2* contributed to downregulated *BMPR2* expression in epithelial and immune cells, at least in part by interacting with cigarette smoke-induced effects. The findings of our study are limited by hospital-based case–control population in southern Chinese. Larger population-based studies in different ethnic groups are warranted for future validation. In addition, this study is also limited by SNP assessment in the 3′UTR of *BMPR2*, the roles of SNPs or other types of mutations in other regions of *BMPR2* gene, i.e. the promoter, the coding exons and introns, etc., worth further investigating to fully understand the relationship of BMPR2 mutation and COPD risk.

In summary, this study demonstrated that the functional polymorphism rs6435156C>T in the 3′UTR of *BMPR2* contributes to increased risk of COPD likely via binding with has-miR-20a. The rs6435156T genotype of *BMPR2* may serve as an important target for novel drug development and as a biomarker for COPD susceptibility assessment.

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Author Contributions

WJL, JW, CTZ, NSZ and ZGZ conceived and designed the experiments; ZGZ, CTZ, ZLZ and QY performed the experiments; WJL, CTZ and ZLZ analyzed the data; NSZ, ZGZ, DJS, DFL, XMX, YFW, CT, MMX, QPZ, MHG, QY and GHX contributed to recruitment of case and control subjects; WJL, CTZ, JW, and ZLZ wrote the paper; WJL, JW, KY, CH, CTZ, ZLZ and DJS revised the paper: All authors reviewed the manuscript.

Conflicts of Interest

The authors declare no financial or commercial conflict of interest.

Acknowledgments

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