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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

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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.

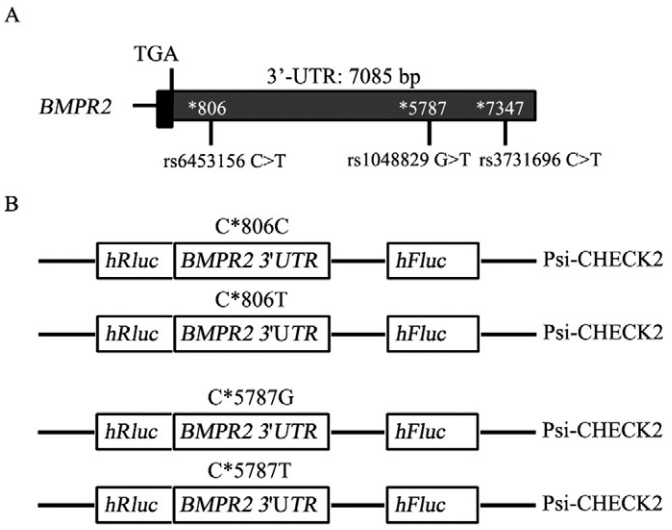

Fig. 1. Genomic structure of *BMPR2* and reporter gene constructs for the 3′UTR of *BMPR2*. (A) Location of the three validated SNPs (rs6435156C>T, rs1048829G>T and rs3731696C>T) in 3′UTR of *BMPR2* gene. (B) Schematic drawing of the reporter gene constructs containing a 618 bp of 3′UTR of *BMPR2* with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

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 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