The SNP rs13118928, rs1828591 and rs10519717 in the HHIP Gene are not Associated on COPD Susceptibility in Male Javanese Smokers

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The SNP *rs*13118928, *rs*1828591 and rs10519717 in the HHIP Gene are not Associated on COPD Susceptibility in Male Javanese Smokers

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Abstract

Background: Hedgehog Interacting Protein (HHIP) gene polymorphisms have an association on COPD has been carried out in Europe and Asia but in Indonesia there is still very limited study on this type and the largest ethnic group in Indonesia is the Javanese. **Objective:** To analyze the association between the HHIP gene polymorphism and the incidence of COPD in male Javanese smokers in Lampung, Indonesia. **Method:** In a case-control study in Javanese male smokers, three single nucleotide polymorphism (SNPs) in the HHIP gene were analyzed by Sanger sequencing method. There were 110 participants in this study which were divided into 2 groups, such as COPD group (55 participants) and control group (55 participants). Three SNPs in the gene (rs13118928, rs1828591 and rs10519717) were selected for genotyping. Genotype distributions were compared between patients and controls. The statistical analysis was carried out with the SPSS program with a chi-square test. **Result:** The genotypic frequency of the HHIP gene sequence at the SNP position rs1828591, such as AA (52.72%), GG (3.63%) and AG (43.63%) in COPD group, while in the control group such as AA (38.18%), GG (9.09%) and GG (52.72%; $p > 0.05$). The genotypic frequency of the HHIP gene sequence at the SNP position rs13118928 consisted of AA (47.27%) and AG (53.72%) in the control group, while the COPD group consisted of AA (52.72%), GG (1.81%) and AG (45.45%; $p > 0.05$). The genotypic frequency of the HHIP gene sequence at the SNP position rs10519717 consisted of TT (34.54%), CC (14.56%) and CT (50.90%) in COPD group, while controls group consisted of TT (23.63%), CC (16.37%) and CT (60.00%; $p > 0.05$). The genotypic analysis of Three SNPs in HHIP gene were observed but showed no significant difference between case and control groups. **Conclusion:** Single-nucleotide variants in the HHIP gene are not associated with COPD susceptibility in Javanese male smokers.

Keywords: *HHIP, COPD, Javanese, Indonesia, male smokers*

Introduction

Chronic obstructive pulmonary disease (COPD) is one of the major health problems that occur worldwide and in Indonesia. This disease is the fourth leading cause of death in the world, causing 3 million deaths of about 80 million people suffering from COPD in 2005. This disease is a major global health problem due to its high prevalence, which is about 10% of the adult population[(1)]. The prevalence of COPD in Asia is estimated at 6.3% with the highest prevalence in Vietnam and the People's Republic of China. The prevalence of COPD

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in Indonesia is not certain. The results of a survey of non-communicable diseases by the Directorate General for the Control of Infectious Diseases & Environmental Health in 5 provincial hospitals in Indonesia (West Java, Central Java, East Java, Lampung, and South Sumatra) in 2004, showed that COPD was the first contributor to morbidity (35%), followed by bronchial asthma (33%), lung cancer (30%) and others (2%). COPD also ranks 6th of the Top 10 Non-Communicable Diseases Causes of Hospitalization in Indonesia in 2009 and 2010[2].

Hedgehog Interacting Protein (HHIP) is an inhibitor of the Hedgehog (HH) signaling pathway and a protein that plays a role in pulmonary tissue remodeling. HH protein is an important morphogen for various developmental processes, including the anteroposterior pattern of the limb and the regulation of left-right asymmetry in embryonic development. The HH signaling pathway plays an important role in pulmonary morphogenesis and cellular response to lung injury. The HH signaling pathway also has implications for cell development, cell repair, and cancer cell development[3]. Several cell surface receptors are responsible for transmitting and/or regulating HH signals. HH protein is an important mediator for smoke-induced diseases such as lung cancer and COPD. HH signaling protein expression is regulated by HHIP. This HHIP protein is a highly conserved and specific HH signaling inhibitor in vertebrates[4]. Expression of HHIP protects mice from cigarette smoke-induced emphysema by reducing the number and activation of lymphocytes in the lungs and reducing the number and size of lymphoid follicles[5].

The HHIP gene is found on chromosome 4 arm of the p locus 13. This gene is highly expressed in the lungs and brain. Several genomic regions that code for HHIP are associated with COPD susceptibility. Polymorphisms can cause changes in gene expression leading to functional changes and ultimately COPD. A significant reduction in HHIP gene expression on mRNA and protein was found in COPD patients compared to smokers with normal lung function[6]. Haploinsufensing the HHIP gene in genetically engineered mice caused an increase in emphysema due to exposure to cigarette smoke. Mice with haploinsufficiency ($HHIP^{+/-}$) are more prone to develop severe emphysema due to exposure to cigarette smoke than wild type mice ($HHIP^{+/-}$)[5].

Study on the polymorphism of the HHIP gene and its association with COPD has been carried out by many researchers, especially in Europe and Asia. A European genome association study has shown that two single nucleotide polymorphisms (SNPs) near the HHIP gene, namely SNP rs1828591 and rs13118928, are associated with the risk of COPD. Both SNPs are significantly associated with the risk of COPD in Caucasians[7]. The Asian study involved Han Chinese and Mongolian populations. The HHIP gene is involved in COPD susceptibility in the Han Chinese population. The polymorphism of the HHIP SNP gene rs10519717 is associated with the severity of COPD[8]. Smokers with the HHIP variant rs7654947 were associated with the development of COPD and decreased lung function. Smoking and gene susceptibility have a cooperative effect on the risk of COPD and decreased lung function[9]. HHIP gene polymorphisms on SNP rs10519717 are associated with COPD susceptibility in Mongolians[10].

The causal association between genetic factors and COPD is still a hot topic of study around the world with varying results. Based on the evidence currently available, it is possible that in the future the role of doctors will shift. Currently the role of doctors is primarily to recognize and manage disease, the main role of doctors in the future is to interpret and apply genetic and genomic information in prevention and therapy[11].

Study on the polymorphism of the HHIP gene and its relationship with the risk of COPD in Indonesia has not been reported. The largest ethnic group in Indonesia is the Javanese, which is estimated to be 41% of the entire population of Indonesia. A preliminary survey at the Harum Melati Respiratory Clinic, Pringsewu Regency, Lampung Province, obtained data on the number of COPD patients in 2019 as many as 161 people from a total of 1600 patients with lung disease. Most of the COPD patients (104 people / 64.5%) came from Javanese ethnicity. Based on the description above,

it is necessary to analyze the relationship between the Hedgehog Interacting Protein Gene Polymorphism and the Chronic Obstructive Pulmonary Incidence in the Javanese Tribe in Lampung, Indonesia.

Method

Participant

One hundred and ten male Javanese smokers were included in a case-control study.These subjects attended the Harum Melati Respiratory clinic, Pringsewu, Lampung. Case group of this study were patients diagnosed with COPD and were active smokers. The inclusion criteria for the group included being diagnosed with COPD[12] with a stable condition, having a history of smoking >10 pack years on the Brinkman index[13] and Javanese (both parents are Javanese). The COPD group exclusion criteria included COPD patients with comorbidities such as tuberculosis, diabetes mellitus, liver and kidney disorders acquired by history and physical examination, COPD patients in exacerbations, and patients not cooperating with spirometry. Meanwhile, The control group consisted of participants who had the following criteria: healthy male smokers, >40 years old, had a history of smoking >10 pack years on the Brinkman index[13], Javanese (both parents were Javanese), and had no history of smoking. COPD, bronchitis, bronchiectasis, bronchial asthma, pneumothorax, lung cancer, bronchopulmonary allergic disease, and no family history of COPD.

Design Study

This study was an observational study with a cross sectional comparative study design. In this study, the participants were divided into 2 groups which included COPD participants who were active smokers and participants who were healthy but active smokers. Sampling of patients was carried out at the Harum Melati Lung Specialist Clinic, Pringsewu Regency, Lampung Province, while the control group was obtained in Pringsewu and Tanggamus districts. Pulmonary function measurements and blood sampling were carried out at the Harum Melati Lung Specialist Clinic, Pringsewu Regency, Lampung Province. DNA extraction and PCR processes for the HHIP gene were carried out at Unit Pelaksana Teknis (UPT) Laboratorium Terpadu dan Sentra Inovasi Teknologi (LTSIT) Universitas Lampung. Sequencing is carried out by a gene sequencing service provider, namely Genetika Indonesia Ltd, Jakarta, Indonesia.

Pulmonary Function Test Using Spirometry

Lung function tests were measured by the spirometry method using a flow spirometer (CHESTGRAPH HI 101; Chest MI, Inc, Tokyo, Japan). Spirometry measures two volumes of air, namely the volume exhaled strongly during maximum inspiration or what is called forced vital capacity (FVC), and the volume of air exhaled in the first second of this breath or Forced expiratory volume in one second (FEV1). A comparison between FEV1 and FVC (FEV1/FVC ratio) is calculated. If the FEV1/FVC value after bronchodilator administration is less than 70%, the lung function test results are declared obstructive.

Genetic analysis

All of the participants' blood was collected into Vacutainer ®tubes. Blood specimens were centrifuged with a special solution, namely Pancoll. This solution is in the form of a sugar solution that has a certain specific gravity, which when the blood is placed on top of this solution, then centrifuged will separate the blood into its component components. Initially 4 ml of Pancoll solution was put in a tube, then carefully 3 ml of blood was placed on top of the Pancoll solution with the help of a pipette. Then the tube rotates using a centrifugator with a speed of 5000 rpm for 10 minutes. Note the buffy coat/yellowish layer over the pancoll layer. With a single-use pipette, collect as much of the yellow layer as possible (generally in <0.5mL volume), avoiding taking significant amounts of plasma or RBCs. Transfer the buffy coat to a sterile pour-off tube.

The process of genomic DNA extraction was derived from 500 µL of buffy coat using a DNA extraction device brand Wizard® Genomic DNA Purification

Kit made by Promega in Madison, USA following the extraction protocol from the manufacturer. The quality and quantity of extracted DNA were analyzed using a nanophotometer made by IMPLEN, Munich, Germany.

In this study, three pairs of primers were designed. The primary source was taken from the sequence "Homo sapiens Hedgehog Interacting Protein (HHIP) gene, complete cds" with access code DQ995342.1. The first pair amplifies the SNP rs1828591, the forward sequence is TGAGGTTGAGTTTGGAG and the reverse sequence is 5'-AGAGGTGTTTCATGTTTCCA-3 '. The second primary pair amplified SNP rs13118928, with a forward 5'-CCCTTCATACCTCCTTCTC-3 'sequence and a 5'- GGTGGGAAGAAACATTACA-3' reverse sequence. The third primary pair amplified SNP rs10519717 with the forward sequence 5'-TACGTGATGTTTTGGGCT-3 'and the reverse sequence 5'- GGTGAACAGACTCCAAACTC-3'.

PCR multiplication was carried out with a total volume of 50 µL consisting of 10 nanograms of sample DNA, each 0.2 micromolar forward and reverse primer, 10 parts Taq Buffer, TaqGold, and 200 micromolar dNTP each.

The electrophoresis process aims to evaluate the PCR results. The PCR results were visualized using a digital electrophoresis device using Qiagen's QIAxcel DNA High Resolution Kit following the electrophoresis procedure from the manufacturer. If there is amplification, a band will appear. Based on the source of the primary sequence taken, namely the sequence with the access code DQ995342.1, SNP amplifying primer rs1828591, will produce a single band of DNA with a size range of 491 bp, primers for rs13118928 will produce single bands of DNA 381 bp, and primers rs10519717 will produce bands single DNA 545 bp. The amplicons was then sent for sequencing. Sequencing was carried out by a gene sequencing service provider, namely Genetika Indonesia Ltd, Jakarta, Indonesia. SNPs analysis was carried out on the SNPs rs1828591, rs13118928, and rs10519717 with bioedit and chromaspro software in both forward and reverse sequences. In electroferogram analysis using bioedit software, the nitrogen base sequence is represented by peaked waves with various colors. Base C is yellow, base G is white, base T is green, base A is pink. Generally, one position has only one nitrogen base, but at one SNP position there can be 2 electroferogram peaks, for example bases A and G. This shows that the site is a heterozygous AG genotype.

Statistical Analysis

The DNA samples of 110 male Javanese smokers with and without COPD were genotyped, and three SNPs (rs13147759, rs1828591, and rs13118928) were evaluated. Genotype distributions were compared between patients and controls. The statistical analysis was carried out with the SPSS program with a chi-square test. Data analysis used IBM SPSS Statistics software version 22.0 (IBM Corp., Armonk, NY, USA).

Results

Participant characteristics

The characteristics of the participants can be seen in Table 1 which is described in detail regarding the differences between the COPD and control groups. There was no significant difference between the COPD group and the control group in each variable related to participant socio-demographic characteristics. There were a significant difference in the lung fuctions (p= 0.000), history of respiratory tract infections at childhood (p = 0.004) and body mass index (p = 0.030).

The Association between HHIP gene polymorphisms in smokers with COPD and Control

The Genotype distributions were compared between patients and controls can be seen in table 2. There were 3 genotype of the HHIP gene sequences in the SNP rs1828591 position, namely AA, GG and AG. In COPD who were smokers, there were 29 cases of GG (52.72%), GG 2 cases (3.63%) and AG 24 cases (43.63%), while in control AA 21 (38.18%), GG 5 (9.09%) and GG 29 (52.72) %). Statistically, it was found that there was no p value < 0.05 in the genotype comparison of the HHIP gene at position rs1828591. This means that there were

no differences in the genotype frequency of the SNP rs1828591 of HHIP gene between COPD smokers and healthy smokers in Javanese in Lampung Province, Indonesia.

In table 2 it can be seen that in the control group there were only 2 genotype of the HHIP gene sequence in the SNP position rs13118928, namely AA and AG. In the control group obtained AA 26 (47.27%), and AG 29 (53.72%), while in COPD group, AA 29 (52.72%), GG 1 (1.81%) and AG 25 (45.45%) were obtained. Statistically, there was no p value <0.05 on the AA, GG and AG genotypes, meaning that there was no difference in the frequency of the SNP rs13118928 HHIP gene polymorphism between COPD smokers and healthy smokers in Javanese in Lampung Province, Indonesia.

In table 2 it can be seen that there are 3 genotype of the HHIP gene sequences in the SNP position rs10519717, namely CC, TT and CT. In COPD who were smokers, there were 19 cases (34.54%), CC 8 cases (14.56%) and CT 28 cases (50.90%), whereas in controls TT 13 (23.63%), CC 9 (16.37%) and CT 33 (60.00) %). Statistically, there was no p value <0.05 in the genotype comparison of the HHIP genotype at position rs1828591. This means that there is no difference in the frequency of the SNP rs10519717genotype of the HHIP gene between COPD smokers and healthy smokers in the SNP rs10519717 genotype in Javanese in Lampung Province, Indonesia.

Table 1. Characteristic of Participants

Variable	Participant		p-Value
	COPD	Control	
Age (years)	64.46	61.62	0.790
Indeks Brinkman (packyears)	378.41	325.90	0.632
FEV1 % (Mean±SD) FCV % (Mean±SD) FEV1/FVC % (Mean±SD)	45.40 ± 20.47 74.50 ± 19.49 60.89 ± 14.51	103.81 ± 20.07 94.64 ± 16.18 110.64 ± 18.64	< 0.001* < 0.001* < 0.001*
Work place Room (n/total) Outdoor (n/total)	 7/55 48/55	 13/55 42/55	0.056
Level of education Low (n/total) Medium (n/total) High (n/total)	 46/55 6/55 3/55	 41/55 11/55 3/55	0.273
Income level Low (n/total) Medium (n/total) High (n/total)	 48/55 5/55 2/55	 47/55 7/55 1/55	0.485

***Cont.*. Table 1. Characteristic of Participants**

Allergy Yes (n/total) No (n/total)	 15/55 40/55	 16/55 39/55	0.839
Upper Respiratory Infection History in Childhood Yes (n/total) No (n/total)	 9/55 46/55	 2/55 53/55	0.004*
IMT (Kg/m2)	21.10	23.20	0.030*
Albumin (g/dl)	3.92	3.91	0.988

Note: *significant if $p < 0.05$

Table 2. Genotype frequencies among study groups.

Genotype	Participant		p-Value
	COPD. n=55 (%)	Control. n=55 (%)	
rs1828591 AA GG AG	 29 (52.72) 2 (3.63) 24 (43.63)	 21 (38.18) 5 (9.09) 29 (52.72)	 0.126 0.438 0.340
rs13118928 AA GG AG	 29 (52.72) 1 (1.81) 25 (45.45)	 26 (47.27) 0 (0.00) 29 (52.72)	 0.567 1.000 0.446
rs10519717 TT CC CT	 19 (34.54) 8 (14.56) 28 (50.90)	 13 (23.63) 9 (16.37) 33 (60.00)	 0.208 0.792 0.337

Discussion

COPD is defined as a disease state characterized by poor progressive and reversible airflow limitation that is usually associated with an abnormal pulmonary inflammatory response. Nearly 90% of COPD is caused by long-term smoking, however, only 25% of smokers end up being COPD patients. In addition, COPD tends to occur more frequently in smokers with a family history of obstructive airway disorders including asthma and COPD. All of this suggests that, apart from smoking, there are other genetic factors underlying the development of COPD[1].

Many factors can contribute to COPD. COPD can be influenced by racial, ethnic, gender and environmental factors as well as genetic factors. The variability found in lung function and the risk of COPD in people with a similar smoking history, together with family aggregation studies, supports the important role of genetics in the pathophysiology of COPD[14].

Genome association studies have identified several genomic regions that are clearly associated with COPD susceptibility. However, despite recent advances in the genetics of COPD, much of the heritability of COPD remains unexplained. To date AAT deficiency is the only well-identified genetic risk factor for COPD. It is strongly suspected that several other genes are involved in this disease process. Variants of several genes have been investigated and identified to have a close association with COPD, such as TLR-9, HHIP, IREB2, CHRNA3/5, and HHIP[7].

Pathophysiology COPD is a multifactorial process with a complex profile of inflammatory cells including eosinophils, macrophages, neutrophils, and lymphocytes. Levels of several cytokines, such as interleukin (IL) 8, interleukin (IL) -6, TNF-α, and VEGF are elevated in stable COPD patients, suggesting their key role in the pathogenesis of COPD. Therefore, COPD develops as a result of multiple steps involving inflammatory cells and mediators, of which local inflammation in the lungs is essential as it affects airway remodeling and parenchymal destruction[1].

The results of this study were the HHIP gene polymorphism had no effect on the incidence of COPD in Javanese. The HHIP protein is an inhibitor of the HH signaling pathway. HH protein is an important morphogen for various developmental processes, including the anteroposterior pattern of the limb and the regulation of left-right asymmetry in embryonic development[15]. HH protein is also an important mediator for diseases caused by cigarette smoke such as lung cancer and COPD. HH protein activity is regulated by HHIP. This HHIP protein is an inhibitor of HH signaling, and it has been shown that HHIP expression protects mice from cigarette smoke-induced emphysema[5].

The HHIP gene is found on chromosome 4 arm of the p locus 13. This gene is highly expressed in the lungs and brain. Several genomic regions that code for HHIP are associated with COPD susceptibility. Polymorphisms can cause changes in gene expression leading to functional changes and ultimately COPD. A significant reduction in HHIP gene expression on mRNA and protein was found in COPD patients compared to smokers with normal lung function[6]. Haploinsufficiency of the HHIP gene in genetically engineered mice caused an increase in emphysema due to exposure to cigarette smoke. Mice with haploinsufficiency ($HHIP^{+/-}$) are more susceptible to developing severe emphysema due to exposure to cigarette smoke than wild type mice ($HHIP^{+/-}$)[5]. In this study, it was found that the presence of polymorphisms in the HHIP gene at positions rs13118928, rs1828591 and rs10519717 did not make Javanese smokers susceptible to COPD[16].

The results of this study differed from several studies in Europe and Asia. Study in Europe has shown that two single nucleotide polymorphisms (SNPs) of the HHIP gene, namely SNP rs1828591 and rs13118928, are associated with the risk of COPD. Study in Asia has concluded that the HHIP gene is involved in COPD susceptibility in Chinese Han populations[8, 9, 17] and in Mongolians[10].

This study is a preliminary finding in pioneering a road map for genetic susceptibility study against

COPD in Indonesia. There is only one publication of the relationship between gene polymorphisms and COPD in Indonesia, namely the study of Tarigan et al[18]. Study on genetic susceptibility to a disease requires a lot of study with a homogeneous sample and needs to be carried out in various tribes. This is because Indonesia is a country with ethnic and ethnic diversity, which of course has a large genetic variation. This study will contribute to the successful prevention of COPD. This study could contribute to the initial screening of individuals who are susceptible to COPD, as well as play a role in early screening that will prevent smokers from developing COPD.

Groups at high risk of COPD could be screened using genetic screening in the future, although much effort must be made in this clinical area. We focus on pulmonary function and airway inflammation in patients with COPD. More comprehensive data should be obtained to reveal the mechanisms of COPD in further studies[11].

However, this study has drawbacks because it does not directly examine the levels of the HHIP protein to determine the genetic expression of the HHIP gene and this study also does not examine other downstream HHIP proteins, such as T lymphocyte activation and degradation of the pulmonary extracellular matrix (ECM) which increases the risk of COPD. This study is a cross-sectional study, so it is not possible to know how often there is a history of infection in COPD patients and how the development of nutritional status in COPD patients.

Conclusion

HHIP SNP gene polymorphisms including rs13118928, rs1828591 and rs10519717 were found in Javanese smokers in Lampung. There is no relationship between the HHIP SNP gene polymorphisms rs13118928, rs1828591 and rs10519717 and the incidence of COPD in Javanese in Lampung. Future studies can examine genes for other genes that may be associated with COPD in Javanese and it is necessary to carry out research on other ethnic groups in Indonesia.

Ethical Approval: Participants have expressed their consent to participate in this study and have signed informed consent. This research has been approved by the Health Research Ethics Committee of the Faculty of Medicine, University of Lampung, Bandar Lampung, Indonesia (2739/UN26/PP.05.02.00/2019).

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Conflict of Interest: The author declare that they have no conflict of interest.

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