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Original Article / *Özgün Makale*

Genotyping and analysis of rs3771150 polymorphism to investigate an association with bronchopulmonary dysplasia incidence in Turkish population

Türk popülasyonunda bronkopulmoner displazi insidansı ile ilişkisinin araştırılması için rs3771150 polimorfizminin genotiplenmesi ve analizi

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ABSTRACT

Objectives: rs3771150 polymorphism on IL-18RAP was chosen to be investigated in terms of determining a relationship with BPD incidence seen in preterm infants born in Turkey.

Materials and Method: Allele and genotype frequencies of this polymorphism was analyzed from genotyping results obtained by multiplex reactions and MALDI-TOF mass spectrometry method. In addition, clinical and demographic data obtained from the patients were also inquired in this study to determine any relationship with BPD incidence.

Results: GG genotype has been determined as the wild type genotype of rs3771150 polymorphism for the Turkish population (n=49 in control group, n=46 in BPD group). When other genotypes (AG and AA) were analyzed no significant relationship was found with BPD incidence (p>0.05).

Conclusion: No significant relationship could be determined between rs3771150 polymorphism and BPD incidence in 192 preterm Turkish infants.

Keywords: Bronchopulmonary dysplasia; multiplex polymerase chain reaction; single nucleotide polymorphism; SNP genotyping.

ÖZ

Amaç: Bu çalışmada Türkiye'de erken doğan bebeklerde görülen BPD insidansı ile IL-18RAP üzerinde bulunan rs3771150 polimorfizmi arasındaki ilişki araştırılmıştır.

Materyal ve Metod: Bu polimorfizmin alel ve genotip frekansları multipleks reaksiyonlar ve MALDI-TOF kütle spektrometresi yöntemi ile elde edilen genotipleme sonuçları kullanılarak analiz edilmiştir. Buna ek olarak hastalardan elde edilen klinik ve demografik veriler de BPD insidansı ile ilişkileri açısından değerlendirilmiştir.

Bulgular: Türk popülasyonu için rs3771150 polimorfizminin yabanıl genotipi GG olarak belirlenmiştir (n=49 kontrol, n=46 BPD hastası). Diğer genotipler (AG ve AA) analiz edildiğinde BPD insidansı ile anlamlı bir ilişki kurulamamıştır (p>0.05).

Sonuç: Erken doğan 192 Türk bebekte rs3771150 polimorfizmi ile BPD insidansı arasında

anlamlı bir ilişki bulunamamıştır.

Anahtar sözcükler: Bronkopulmoner displazi; multipleks polimeraz zincir reaksiyonu; tek nükleotid polimorfizmi; SNP genotipleme.

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Bronchopulmonary dysplasia (BPD) is one of the most encountered lung diseases seen in preterm infants.[1] Lung development and maturation histologically occurs in five phases; embryonic, pseudoglandular, canalicular, saccular and alveolar phases.[2,3] Bronchopulmonary dysplasia disease is seen mostly in preterm infants born between the 23rd-28th weeks of gestation which is the late canalicular/early saccular phase of lung development.[4] Between the 16th and 25th week of pregnancy, fetal lungs are in the canalicular phase of development. During the canalicular phase, with the vascularization of airways and angiogenesis, the respiratory tree grows both in diameter and length, a vast amount of increase occurs in the number of capillaries. Afterwards, terminal bronchioles divide into respiratory bronchioles and alveolar sacs while airway epithelial cells differentiate into peripheral squamous cells and proximal cuboidal cells.[5] The saccular stage starts in the 24th week of pregnancy and continues til the late fetal phase. During the saccular stage, as a result of apoptosis and differentiation of mesenchymal stem cells, interstitium becomes notably thinner.[6,7] Also alveolar epithelial cells differentiate into type 1 secretory pneumocytes and type 1 adult squamous pneumocytes which carry lamellar bodies containing surfactant. Furthermore, capillaries rapidly expand and spread over the sacs (saccule) placed in the mesenchyme forming a complex web. In addition, the lymphatic web of the lung tissue also develops in this phase. Fetal lungs of premature infants born by the end of saccular phase, become mature enough to ensure air interchange. Maturation of the surfactant synthesis and the amount of secretion are the key factors that determine the ability of air interchange without collapsing the lungs of a newborn.[5,8] Lungs of infants born in this phase pause developing and the number of alveoli stays lower than mature babies. Today, interruption of alveoli development, surfactant deficiency and dysmaturity of lungs caused by inflammation are seen as the major etiological factors of BPD.[8-11] While the rate of BPD incidence can increase up to 70% in infants born before the 26th week, the ratio of BPD incidence in infants born after the 34th week decreases to 1%.[12] Whether there is a surfactant deficiency or not, the main reason of BPD is immature lungs and it is thought that 50-80% of predisposition to BPD has a genetic tendency. When the immature and BPD predisposed lung is exposed to harmful exogenous factors like prenatal or postnatal infections, hyperoxia, ventilation induced barotrauma, alveolar distension or atelectrauma, an inflammation chain is triggered by various cytokines, chemokines, growth factor signals and inflammatory cells. This situation activates apoptotic pathways.[13,14]

There have been numerous studies focused on the relationship between certain polymorphism sites and BPD disease reported in the literature. To achieve this, the information of target genes and pathways has to be obtained from experimental models or previous human studies. Thus target genes and pathways which have a higher probability to have a significant relationship can be identified based on biological theories explaining functional relationships between certain genes and proteins encoded by these genes and structural alterations like lung development disorders seen in BPD. For instance in two recent studies, the relationship between BPD and interleukin-18, which is an important regulator of hereditary immune response, has been analyzed. In one of these studies Floros et al.[15] investigated 6324 SNP sites positioned on 601 different genes in 1,091 Caucasian and African-American preterm infants diagnosed with BPD, born before the 35th week of pregnancy in between the years 1989 and 2008. They chose these polymorphisms regarding previous studies reporting that these genes might be involved in developing inflammation and lung diseases. Only two out of 6,324 polymorphisms [rs3771150 (IL-18RAP) and rs3771171 (IL-18R1)] showed a significant relationship with BPD incidences seen in African-American preterm infants in this study (n=98; $q<0.05$). T allele of rs3771150 polymorphism and G allele of rs3771171 polymorphism were identified as possible risk alleles of BPD. However, in another recent study, the relationship between six different polymorphisms on IL-8 gene and typical pulmonary diseases, such as BPD, were investigated in a total number of 574 preterm infants born in Germany, before the 32nd week of pregnancy and were in need of oxygen support after 36th postmenstrual week.[16] Contrasting Floros et al's[15] study, this study's results showed no significant relationship between IL-8 polymorphisms and BPD incidence.

IL-18RAP region polymorphisms are reported to be associated with certain immune-mediated diseases such as inflammatory bowel disease (IBD),[17] atopic dermatitis,[18] leprosy,[19] celiac disease[20] and type I diabetes.[21] Polymorphisms on IL-18RAP and IL-18R1genes are also suspected to be contributing BPD pathogenesis via inflammatory-mediated processes.[15]

By taking these studies into consideration, rs3771150 polymorphism on IL-18RAP was chosen for investigation in terms of determining a relationship with BPD incidence seen in preterm infants born in Turkey. To achieve this, allele and genotype frequencies of rs3771150 polymorphism was analyzed from genotyping results obtained by multi step experiments and Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometry (MS) methods. In addition, clinical and demographical data obtained from the patients were also inquired in this study to determine any relationship with BPD incidence. Our study aims to contribute to the studies intended to understand the genetic basis of BPD in the Turkish population.

MATERIALS AND METHODS

Study population

A total of 192 preterm infants born before the 32nd week of pregnancy in Turkey were included in the study population. Ninety-six of these infants were diagnosed with BPD in Kanuni Sultan Süleyman Training and Research Hospital Neonatal Intensive Care Unit. Bronchopulmonary dysplasia was diagnosed if the infants were in need of <30% oxygen support (mild BPD) or in need of ≥30% oxygen support or positive air pressure via nasal continuous positive airway pressure (nCPAP) or positive pressure ventilation (PPV) (severe BPD). Peripheral blood samples were obtained from these infants by the hospital's neonatal intensive care unit doctors in regard to the approval of research ethics committee of Kanuni Sultan Süleyman Training and Research Hospital (KAEK/2016.12.31).

Chemicals, enzymes and kits used

DNA isolation from whole blood samples obtained from the patients was made using a QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany); HotStarTaq plus DNA polymerase (5 U/µL), 10X polymerase chain reaction (PCR) buffer and MgCl2 (25 mM) solutions used in PCR were obtained from Qiagen, dNTP solution (100 mM) was obtained from Applied Biosystems; ultra-pure water used in all steps was obtained from Invitrogen (Invitrogen, Carlsbad, CA, USA). Shrimp alkaline phosphatase enzyme (1.7 U/µL) and enzyme buffer solution for cleaning PCR products; iPLEX enzyme, buffers and extension reaction mix (Agena Bioscience Inc., San Diego, USA), 10X iPLEX buffer solution and iPLEX Extension Mix used in single base extension reactions and SpectroClean Resin solution used to get samples ready for mass spectrometry analysis were all obtained from Agena Biosciences. Primers used in PCR were synthesized by Metabion.

Methods

Unless otherwise specified, all methods used in this study were carried out according to iPLEX® Gold Application Guide.[22]

Isolation and determining the concentration and purity of genomic DNA

DNA isolation from whole blood samples was done according to the protocol of the kit.[23] Concentration and purity of the isolated DNA samples were determined by spectrophotometric measurements at 260 nm and 280 nm wavelengths. DNA samples with an absorbance ratio of 1.7-2 (A260/A280=1.7-2.0) were included in the study.[24] Concentrations of the DNA samples were calculated according to the formula below:

DNA amount= OD unit x dilution factor x 50 µg/mL.[1]

SNP genotyping

iPLEX method was used to genotype rs3771150 polymorphism in this study. In this method, the genomic sites that the polymorphisms are involved in are amplified by using primers designed specifically for these sites via PCR. Afterwards, single base extension reactions are done using PCR products as templates. After an extension primer binds to the 3' end of the sequence of the PCR product, this primer is extended with a single base. PCR product which includes the SNP site is used as a template and the primer's 3' end consists of the allelic base. Extension of the primer only happens if the 3'

Table 1. Single base extension reaction conditions

Steps	Process	Temperature and time
1	Pre-denaturation	94°C 30 seconds
2	Denaturation	94°C 5 seconds
3	Annealing	52°C 5 seconds
4	Extension	80°C 5 seconds
5	Final extension	72°C 3 minutes

base is complementary to the allele on the target DNA. Following the extension of the primer allows us to understand which allele the DNA sample carries.[21,22] Target site on the genomic DNA has been amplified by PCR using forward and reverse primers as shown in Table 1. PCR and single base extension reaction primers are explained in Table 2. Determination of the polymorphism allele is done by measuring the mass of the extended nucleotide via MALDI-TOF mass spectrometry. Amplification reaction steps were carried out as follows, pre-denaturation step for two minutes at 92°C, 45 cycles of denaturation for 30 seconds at 95°C, annealing for 30 seconds at 56°C and extending for one minute at 72°C, and a last extension step for three minutes at 72°C. Shrimp alkaline phosphatase (SAP) was used to neutralize dNTP's which are not bonded to the amplification products by cutting the free phosphate group from dNTP's and turning them permanently into dNDP's. 2 µL SAP solution was prepared with 0.30 µL SAP enzyme (1.7 U/µL), 0.17 µL TS Buffer (10X) and 1.53 µl dH_2O and added to the PCR products. Shrimp alkaline phosphatase reaction was carried out at 37°C for 40 minutes and after that the enzyme was inactivated by incubation for five minutes at 85°C. Shrimp alkaline phosphatase reaction products were then hybridized with designed extension primers and single mass-modified base extension reaction was performed. Reaction mixture was prepared with 0.619 µL dH_2O, 0.2 µL iPLEX buffer (10X), 0.2 µL iPLEX termination mixture, 0.940 µL iPLEX extension primer mix and 0.041 µL iPLEX enzyme and 2 µL of this single base extension reaction mixture was added to samples.[22] Single base extension reaction conditions are given in Table 1. Single base extension reaction products were treated with resin to remove Na^+, K^+ and Mg^{2+} ions to reduce background noise. The modified PCR product obtained was then transferred to 384-element SpectroCHIP® II via a nanodispenser.

Table 2. DNA sequence that rs3771150 polymorphism is involved (forward primer is designed according to the sequence shown in red, reverse primer in green and extension probe sequence is underlined)

DNA sequence that involves rs3771150 polymorphism	Length
5'GGGTTTAAACCCATTTTCTATCTG ATGTCAACATGACCCTTAGCCCCGGT [A/G]AGCCTCCATCACCCATTGATAAT CTATGGATAATAGCCTGCACTTTGCAA3'	50 bp [A/G] 50bp

Finally the SpectroCHIP® II was loaded to the mass spectrometry device (MassARRAY® Analyser 4) to be analyzed. MassARRAY® TYPER 4.0 genotyping software was used to analyze data, obtain allele specific peaks and spectro images.[22]

Statistical analysis

IBM SPSS for Windows version 20.0, (IBM Corp., Armonk, NY, USA) software was used to perform statistical analyses. Statistical significance was approved if $p \leq 0.05$ in all tests. Chi-square test was used to evaluate Hardy-Weinberg equilibrium. To determine the relationship between BPD and SNP alleles, the major allele seen in the control group was referred to as the wild type allele of the polymorphism. The odds ratio (OR) of the minor allele which is thought to have a relationship with the disease in reference to the wildtype allele was calculated with 95% confidence interval. The association between SNP genotypes and BPD was determined referring the common homozygote genotype seen in control group. Odds ratio values and confidence interval were calculated according to homozygote comparison model (e.g., AA and CC) and dominant model (e.g., AA+AC and CC). Chi-square and Fisher exact test were used to determine a relationship between the polymorphisms and clinic-pathological parameters such as, other medical history of premature birth, chronic lung diseases, pneumonitis, tuberculosis, RDS, BPD seen in siblings.

RESULTS

Concentration and purity of DNA isolated from patient's whole blood

Spectrophotometric analyses at 260 and 280 nm wavelengths showed that all isolated DNA

Table 3. Allele and genotype frequencies of rs3771150 polymorphism

rs3771150	BPD group (n=90)	Control group (n=91)	OR (95% CI)	*p*
Allele level				
G	0.728	0.753	Reference	
A	0.272	0.247	1.139 (0.93-1.39)	0.2
Genotype level				
GG	46 (0.511)	49 (0.538)	Reference	
AG	39 (0.433)	39 (0.428)	1.065 (0.58-1.93)	0.83
AA	5 (0.055)	3 (0.032)	1.775 (0.40-7.85)	0.44
AG + AA	44 (0.488)	42 (0.461)	1.115 (0.62-2.00)	0.71

samples (192 samples) had an A260/A280 ratio between 1.7 and 2.0. Also DNA concentrations of all samples were higher than 10 ng/μL, therefore all DNA samples were included in the following experimental steps.

Genomic position of rs3771150 polymorphism and primers

The physical position of rs3771150 polymorphism in the genome is determined as 102,444,391, its chromosomal location was designated as 2q12.1 on IL-18RAP gene and its alleles were specified as C/T.[15,16] The positions of primers and extension probe on the genome sequence were determined manually. Finally a bioinformatics software was used to verify the locations of primers and the extension probe (Table 2).

Both of the forward and reverse primers have been designed with a 10-mer label (5'-ACGTTGGATG-3') attached to the 5' end of them (forward primer: 5'' and the reverse primer as:5'ACGTTGGATGGCAAAGTGCAGGCTAT TATC3'). As seen on Table 2, the underlined extension probe sequence's 3' end is right at the polymorphism site.

Genotyping studies have been performed for rs3771150 polymorphism in 192 DNA samples. Ninty BPD and 91 control samples were successfully genotyped. Chi-square tests for Hardy Weinberg equilibrium showed that the study population for rs3771150 polymorphism was in balance (p=0.104). When analyzed at genotype level, GG genotype has been determined as the wild type genotype for the Turkish population (n=49 in control group, n=46 in BPD group). When other genotypes (AG and AA) were analyzed no significant relationship was found with BPD incidence (p>0.05). When analyzed at allele level, the frequency of G allele was 0.728 in BPD group and 0.753 in the control group; the frequency of A allele was 0.272 in BPD group and 0.247 in the control group. Although odds ratio value was calculated as 1.139 (95% CI= 0.93-1.39) when BPD and control groups were compared, *p* value was calculated as 0.2 (Table 3). In addition, chi-square and Fisher exact tests were also performed to investigate any relationship between rs3771150 polymorphism and clinical and demographical data obtained from the patient's family.

DISCUSSION

No significant relationship could be determined between rs3771150 polymorphism and BPD incidence in the Turkish population (Table 3). Also when clinical and demographical data was analyzed for a relationship with BPD incidence, we could not find any significant relationship between rs3771150 polymorphism and other medical history of premature birth, chronic lung diseases, pneumonitis, tuberculosis and RDS in family and BPD history seen in patient's siblings (p>0.05).

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Declaration of conflicting interests

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

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