Allelic frequency of PAR-2 in the Puerto Rican population: a possible insight to asthma prevalence l

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Resumen

El Proyecto del Genoma Humano secuenció el componente genético de nuestra especie: el DNA. Dicha secuenciación se realizó empleando al azar DNA de individuos de diversos orígenes étnicos. Esta selección aleatoria no garantiza que todas las poblaciones se encuentren igualmente representadas en esa "secuencia promedio" generada y publicada. Existen variaciones genéticas de DNA conocidas como polimorfismos. Comúnmente los científicos emplean los polimorfismos para explicar la diferencia de enfermedades complejas en ciertas poblaciones. En este estudio, al analizar la población puertorriqueña se identificaron seis polimorfismos (dos noveles) en una región de mil bases pareadas del receptor PAR-2. Este receptor previamente caracterizado en poblaciones de descendencia africana y europea no había sido estudiado en poblaciones hispánicas. La población muestra un valor marginalmente significativo (p=0.06) en la prueba de Hardy-Weinberg, sugiriendo alteraciones en la ley de sorteo independiente de genes. Se evalúa además la posibilidad de utilizar el PAR-2 como marcador de predisposición a asma.

Abstract

The Human Genome Project has sequenced the genetic complement of our species: the DNA. Such sequencing was performed using DNA randomly collected from individuals of diverse ethnicity backgrounds. This aleatory selection does not warrant that all existent populations are equally represented in the generated and published "consensus sequence". There are DNA genetic variations known as polymorphisms. Commonly scientists employ polymorphisms to explain the occurrence of complex diseases in a certain population. In this study, by analyzing the Puerto Rican, we identified six polymorphisms (two novels) within a region of one thousand paired bases of the PAR-2 receptor. Although previously characterized in population of African and European descent, up to now, it hasn't been studied in Hispanic populations. The studied population showed a borderline significant value (p=0.06) in the Hardy Weinberg test, suggesting alterations to the gene independent assortment law. We also evaluate the possibility of employing PAR-2 as an asthma predisposition marker.

BACKGROUND

Asthma in Puerto Rico

Puerto Rico shows the highest lifetime prevalence of asthma in the USA and its territories (Pérez-Perdomo, *et al.*, 2003), ranging from 13-18% (Montealegre and Bayona, 1996; Pérez Perdomo, *et al.*, 1999). It is unknown whether the degree and severity of asthma in the Puerto Rican patients is due to the presence and extent of inhaled indoor environmental irritants, their potential interaction with candidate gene products, or if gene-environment interactions are equally affected by behavioral and health care risk factors.

Asthma genetics and Biological effects of PAR-2 activation

The underlying causes of asthma remain uncertain, although genetic as well as environmental factors have been proposed to contribute in its clinical manifestations. At least nineteen genetic loci have been identified as predisposing factors in this complex disease (Barnes and Marsh, 1998; Orban and Gibson, 2004). Studies have agreed on the contribution of a group of proteins with catalytic activity known as "proteases", as triggers of the inflammatory response. A G-protein-coupled receptor called Protease Activated Receptor 2 (PAR-2), has been found expressed in human respiratory airway epithelial cells, human bronchial vessels and epithelial cells, human airway smooth muscle, eosinophils, neutrophils, basophils and mast cells (Bolton, *et al.*, 2003; Uehara, *et al.*, 2003; Ubl, *et al.*, 2002; Berger, *et al.*, 2001; Chambers, *et al.*, 2001; D'Andrea, *et al.*, 2000). All these cells and tissues are well known to be involved in asthma pathogenesis. Interestingly, the gene encoding PAR-2 is located at chromosome 5q13.0, near one of the previously identified asthma candidate loci (Orban and Gibson, 2004).

PAR-2 promotes an inflammatory response, which can lead to asthma attacks (animal and *in vitro* studies by Kawabata, 2002). Other studies that used animal models have shown that environmental trypsin and mast cell tryptase can cause bronchoconstriction via the PAR-2 (Ricciardolo, *et al.*, 2000; Berger, 1999). Allergic mice exposed to trypsin, increased the expression of PAR-2, which results in eosinophil infiltration, hyperactivity, and inflammation of the airway (Schmidlin, *et al.*, 2002). These findings were particularly relevant to our research because when trypsin and other environmental proteases are inhaled, they can provoke the asthmatic symptoms by activating PAR-2. *In vitro* studies have shown that the dust mite-derived serine proteases *Der p3* (chymotrypsin) and *Der p9* (collagenase/metalloprotease) also interact with PAR-2 and that it induces a non-allergic inflammatory response in airways through the release of pro-inflammatory cytokines (Sun, *et al.*, 2001; King, *et al.*, 1998). It was also demonstrated that *Der p 1*, which is a cysteine-protease, induced the production of IL-6 and IL-8 in the A549 cell line which expressed PAR-2, but not PAR-1 (Asokananthan *et al.*, 2002a; 2002b). Moreover, Knight *et al.* (2001) have demonstrated that PAR-2 expression in asthmatic bronchial epitheliums was significantly increased in comparison with normal epitheliums. These results together supported the hypothesis that environmental exposures

to proteases may have important implications in asthma symptoms and therefore, the study of the PAR-2 receptors at the DNA level was highly justified.

However, beside this report, no other human PAR-2 genetic variation analysis associating this receptor with the world incidence of asthma has been carried out. This approach is known as "Single Nucleotides Polymorphisms" (SNP's). Currently, the SNP's are extensively employed to provide physical landmarks for identifying genes related to complex human diseases. More importantly, certain PAR's family members (PAR-1) SNP's have been found only in specific ethnic groups, explaining the occurrence of certain diseases in specific populations (Barnes et al., 2004; Arnaud et al., 2000). In this study, we evaluated the PAR-2 polymorphisms as possible genetic marker for the development of asthma. We analyzed a 1Kb (1,000 base pairs) region of DNA sequences that encode the PAR-2 gene in: 1) regulatory region of Exon/Intron boundary; 2) the activating or proteolytic cleavage site and, 3) the II extra cellular loop domain. We hypothesized that the allelic frequency of PAR- 2 polymorphisms will be statistically higher in Puerto Ricans when compared with other populations. This approach could be eventually used to relate to the high prevalence of asthma in Puerto Ricans with the polymorphisms.

MATERIALS AND METHODS

Sample size and population

We analyzed one hundred Puerto Ricans (n=100) over 21 years old and residents of the southern region of the Island.

Studied population and patient recruitment

The controls (asthma-free participants) were recruited within UPR-Ponce students that were over 21 years old, and asthmatic patients DNA's were provided by the Immunochemistry laboratory at Ponce School of Medicine (Dr. Federico Montealegre).

Inclusion and exclusion criteria

Asthma patients who participated were validated by medical diagnosis of asthma as evidenced by the clinical criteria according to the National Heart, Lung, and Blood Institute (NHLBI) (Epton, et al., 1999). Candidates were excluded if one or more of the following conditions were diagnosed by their physician: 1) chronic bronchitis; 2) pulmonary emphysema; 3) congestive heart failure; 4) cough secondary to drugs such as beta-blockers; 5) chronic obstructive pulmonary disease; or 6) any other chronic and inflammatory medical conditions restricting their daily activities.

Blood sampling

Volunteers were asked to donate 10 ml of venous blood for analysis. Two 5 ml tubes (5 ml each) were obtained in EDTA tubes and were used to isolate DNA for the genetic studies. A registered nurse in Ponce School of Medicine, as a collaborative effort from Dr. Federico Montealegre, performed the blood draw.

Consent process

Candidates were informed as to the details of the study at this point. Participants who were interested in joining the project were asked to make an assessment visit to the Ponce School of Medicine's Immunochemistry Laboratory and Asthma Research Unit.

During the visit, the persons were asked to a) read and sign the consent form approved by the PSM-Internal Review Board, b) answer a questionnaire, and c) give a blood sample in order to isolate the DNA.

Patient Confidentiality

The required consent forms were approved by Ponce School of Medicine Internal Review Board (IRB). The accessibility to the participants' (patients and controls) confidential information by laboratory technicians was limited to age. A number encoded this information. Other identifiers (name, address, phone, etc.) were not included in the questionnaire. Patient documentation is locked in a file cabinet at Ponce School of Medicine and its access is restricted to authorized personnel only.

DNA Extraction

The DNA extraction was performed with the QIAamp DNA extraction Kit (QIAGEN, Inc. Hercules CA.) and following manufacturers' instructions.

DNA concentration:

The recently acquired Eppendorf Biospectophotometer, was used to calculate the concentration and purity (260/280) of the DNA.

PCR with PAR-2 Exon 2 Primers:

PCR amplification from the participants DNA samples, were performed using the previously designed PARE2b with the Triple Master PCR Kit (Eppendorff Inc. Hamburg, Germany) in a PCR reaction volume of 20 µl: 3.2 µl 10X High Fidelity PCR Buffer, 0.4 µl of

each 10nM DNTP, 0.5 µl of each primer (10 mM), 0.3 µl of TM enzyme, X µl of water (up to 20 l and 100 ng of extracted DNA. PCR conditions were set in an Eppendorf Gradient Mastercycler as follow: denaturation for 5 min. at 94°C and 35 cycles of amplification using a step program of 20 sec at 94°C, 20 sec at 56.0 °C, 5 min at 72°C, a final extension of 10 min at 72°C and a hold step at 4°C. Positive and negative controls were included in each reaction using human reference DNA and water as templates, respectively. PCR products (9µl) were electrophorased in a 2% agarose gel with TAE buffer at 100 volts for 90 minutes, stained with ethidium bromide, and visualized under UV light. This step confirmed the viability of the extracted DNA for downstream applications.

DNA sequencing

The tested positive (+) DNA were diluted up to 10 ng and sent in aliquots of 30 l. The DNA changes were identified by using the Big Dye terminator Protocol at Polymorphic DNA Technologies. Once the sequences were generated, they were sent via email. We performed the sequences edition and analyses by using the BioEdit Software.

Statistical analysis

Data analyses were performed using the software SPSS. The significance level was established at $p<0.05$. The Hardy-Weinberg equilibrium was tested by X^2 test with 1 df in the whole population (100), in cases (69), and controls (31) separately. Allele frequencies were deducted from the genotype frequencies. The allelic frequency data for comparison with the African and European descent

populations was retrieved from: http//pga.gs.washington.edu/data/f2rll/f2rll.ColorFasta.html.

RESULTS

Six mutations were identified, two of which were not previously reported (Table 1). The studied population showed a statistically border line result when tested for the Hardy Weinberg (HW) equilibrium (p=0.06) (Table 2).

| Allele | Puerto Ricans (n=100) | Previous Reports | |
		African Descent (n=26)	European Descent (n=29)
15777	0.40	0.52	0.43
15810	0.08	0.04	0.22
15954	0.02	0.01	0
15971*	0.005	0	0
16646*	0.02	0	0
16684	0.02	0.04	0

Table 1: Allele frequencies comparison with previously reported population; numbers denote the nucleotide position at the DNA reference sequence.

Total				
Total number of individuals				100
Genotypes	TT	TC	CC	3
Total observed	40	39	21	100
Total expected	36	48	16	100
Alleles	A	T		2
Observed allelic frequency %	60	40		100
Chi-square			3.64	
One sided p value (1df)			0.06	

Table 2: Hardy Weinberg testing for allele 15777 in 100 Puerto Ricans. All other five polymorphisms were tested as well, and showed not significant deviation from expected results (p>0.05).

DISCUSSION AND CONCLUSIONS

By analyzing a 1,000 base pairs (1Kb) region encoding the PAR-2 gene at the regulatory region of the Intron/Exon2 boundary, the enzymatic cleavage site, and the II loop domain in 100 Puerto Ricans, we identified six mutations: a T to C transition at position 15777; a G to A transition at position 15810; an A to G transition at position 15954; a T to C transition at position 15971; a C to T transition at position 16646; and a G to A transition at position 16684. Although three of the mutations were identified at the intron/exon boundary region, no mutations were located at the protease cleavage site or at the second loop domain. However, the generated data allowed the identification of two novel polymorphisms not previously reported: 15971 and 16646. The studied population was borderline significant for HW equilibrium. This result implies that a mechanism other than randomness, influenced the allele distribution of the studied population. In other words, it does not follow the gene independent assortment law. In this case, due to the fact that Puerto Rico is an island with several ethnic backgrounds, the founder effect might be influencing the allele distribution. To exclude this possibility, an increase of the sample size and stratification of the population would be required. If founder effect is responsible for the resulting allele distribution, then no differences in such frequency would be expected between cases and controls. This analysis could be the key to explain the high asthma prevalence in Puerto Ricans. Due to the complexity of asthma, our results might provide some insights about the possible protective effect of these polymorphisms on controls and predisposition effect for

asthma suffering patients.

ACKNOWLEDGEMENTS

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