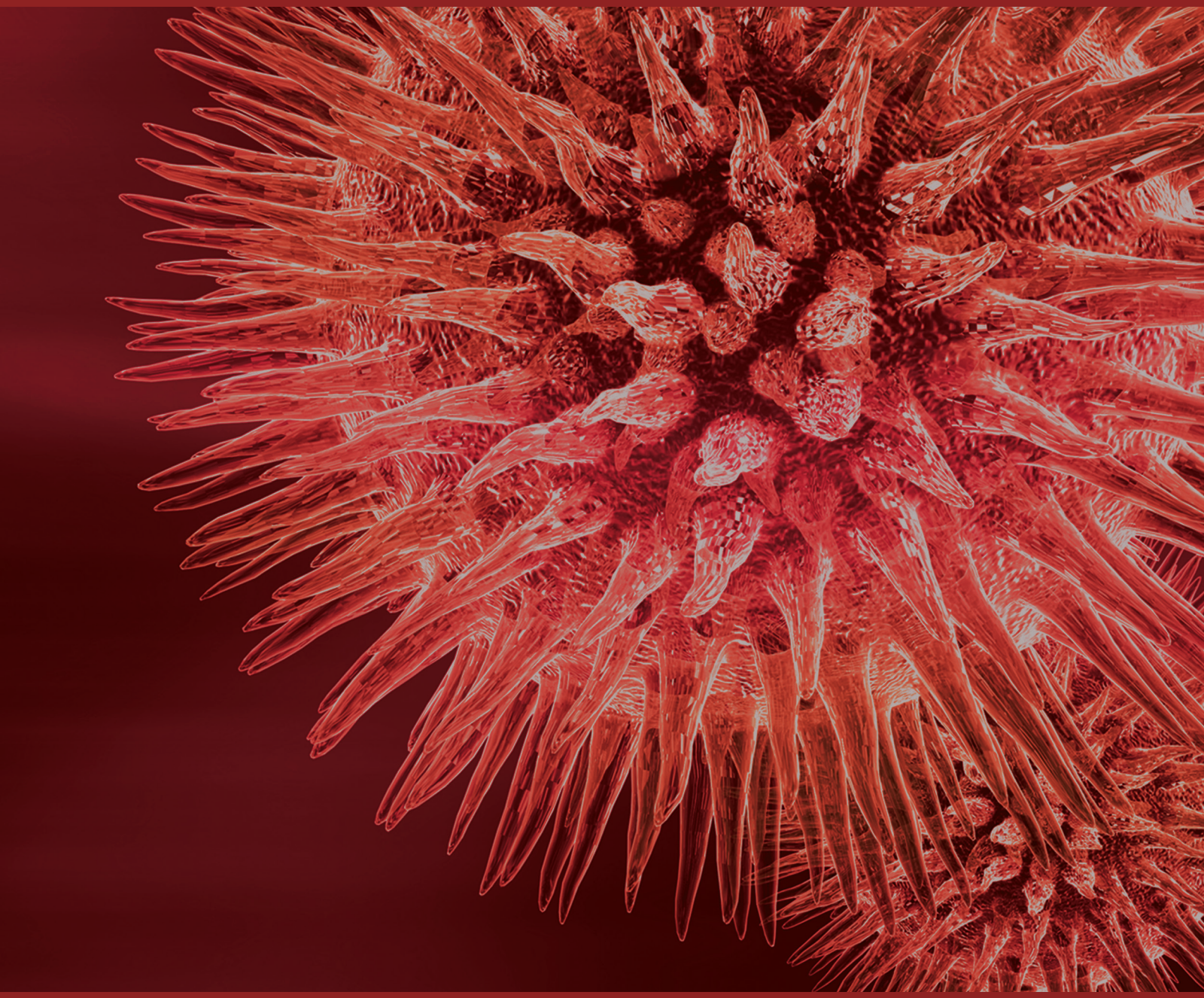
BioMed Research International

Biomedical Insights of Human Genetic Diversity in Complex Diseases

Guest Editors: M. Esther Esteban, Analabha Basu, Carla M. Calò, and Pedro Moral



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Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 974809, 2 pages
http://dx.doi.org/10.1155/2015/974809

Editorial

Biomedical Insights of Human Genetic Diversity in Complex Diseases

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Received 2 February 2015; Accepted 2 February 2015

Once some of the major goals in the understanding of human gene diversity are attained, we have now the opportunity to apply them to the field of biomedicine. The knowledge of complete genomes allowed us to go more deeply into the genetic factors of many diseases. Much effort has been devoted to unravel the genetic and environmental contribution to complex diseases, not without difficulty because they represent the extreme manifestation of a continuum of genetic, physiological, and environmental features. With the added pressure that some of these disorders have reached epidemic proportions because of the lifestyle changes of worldwide populations.

Within this scenario, this special issue contains five works about complex diseases, including heart pathologies, rheumatoid arthritis, colorectal cancer, and childhood asthma. All are multistep diseases with unknown interactions among environmental agents and genetic susceptibility. In addition, their incidences do not stop increasing in both western and emerging countries. The methodological approximations of the five articles respond to different designs including miRNAs binding sites, mitochondrial heteroplasmy, SNP genotyping, and a very particular sample. In the paper entitled "Mosaicism of Mitochondrial Genetic Variation in Atherosclerotic Lesions of the Human Aorta," the samples are segments of morphologically mapped aortic walls instead of human patients as in the remaining papers. In this work, M. A. Sazonova et al. examine the heteroplasmy levels of eleven mitochondrial mutations in segments of morphologically mapped aortic walls, normal and affected by atherosclerosis segments of morphologically mapped aortic walls. Five mutations have been found significantly associated with atherosclerotic lesions of intimal segments.

The next paper concerning heart diseases is entitled "*Novel Mutations* in the Transcriptional Activator Domain of the Human TBX20 in Patients with Atrial Septal Defect." The work conducted by I. E. Monroy-Muñoz et al. analyzes patients affected by congenital heart defects for TBX20 mutations. Three missense mutations located in exons encoding the transcriptional activator domain have been detected, together with other ten nonsense mutations and one nonreported SNP.

The work entitled "*TRAF1/C5* but Not *PTPRC* Variants Are Potential Predictors of Rheumatoid Arthritis Response to Antitumor Necrosis Factor Therapy" examines the association between risk variants of rheumatoid arthritis and response to treatment with antitumor necrosis factor. In their paper, H. Canhão et al. examine more than six hundred Portuguese and Spanish patients and fail to replicate the previously reported association of *PTPRC* locus and response to treatment.

The relationship among risk of asthma, environmental exposures, and genetic background has been deeply examined in the paper entitled "Gender-Dependent Effect of GSTM1 Genotype on Childhood Asthma Associated with Prenatal Tobacco Smoke Exposure." C.-C. Wu et al. conducted a longitudinal birth cohort study of six years of follow-up recruiting more than five hundred children to explore the interactive influences of gender, GSTM1 genotypes, and prenatal tobacco smoke exposure in asthma development.

Finally, the paper entitled "A Functional Variant at miR-520a Binding Site in PIK3CA Alters Susceptibility to Colorectal Cancer in a Chinese Han Population" investigates the association between miR-520a binding site polymorphism in the PIK3CA gene and risk of colorectal cancer. L. Ding et al. conclude that polymorphisms in untranslated regions of PIK3CA may play a role in colorectal carcinogenesis.

Acknowledgment

We would like to thank the authors for their great contribution and the reviewers for their valuable help.

M. Esther Esteban
Analabha Basu
Carla M. Calò
Pedro Moral

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 825468, 9 pages
http://dx.doi.org/10.1155/2015/825468

Research Article

Mosaicism of Mitochondrial Genetic Variation in Atherosclerotic Lesions of the Human Aorta

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Received 25 July 2014; Revised 18 September 2014; Accepted 28 October 2014

Academic Editor: María E. Esteban

Objective. The aim of the present study was an analysis of heteroplasmy level in mitochondrial mutations 652delG, A1555G, C3256T, T3336C, 652insG, C5178A, G12315A, G13513A, G14459A, G14846A, and G15059A in normal and affected by atherosclerosis segments of morphologically mapped aortic walls. *Methods.* We investigated the 265 normal and atherosclerotic tissue sections of 5 human aortas. Intima of every aorta was divided according to morphological characteristics into segments with different types of atherosclerotic lesions: fibrous plaque, lipofibrous plaque, primary atherosclerotic lesion (fatty streak and fatty infiltration), and normal intima from human aorta. PCR-fragments were analyzed by a new original method developed in our laboratory on the basis of pyrosequence technology. *Results.* According to the obtained data, mutations G12315A and G14459A are significantly associated with total and primary atherosclerotic lesions of intimal segments and lipofibrous plaques ($P \leq 0.01$ and $P \leq 0.05$, accordingly). Mutation C5178A is significantly associated with fibrous plaques and total atherosclerotic lesions ($P \leq 0.01$). A1555G mutation shows an antiatherosclerotic effect in primary lesion in lipofibrous plaques ($P \leq 0.05$). Meanwhile, G14846A mutation is antiatherogenic for lipofibrous plaques ($P \leq 0.05$). *Conclusion.* Therefore, mutations C5178A, G14459A, G12315A, A1555G, and G14846A were found to be associated with atherosclerotic lesions.

1. Introduction

Atherosclerosis of great vessels in the overwhelming majority of cases is predominantly a morphological basis of cardiovascular mortality [1, 2]. In the twenty-first century, atherosclerosis began to be of epidemical nature [2]. Previously, this pathology was specific only for elderly people, but now it affects even young individuals. The significance and timeliness of investigation of the pathology seem obvious.

A specific feature of atherosclerosis is a complicacy of this disease detection at early stages. Molecular-genetic markers associated with atherosclerosis can help early diagnosis of this pathology. In recent times, a great number of studies were dedicated to searching genetic biomarkers of atherosclerosis and CVD in human nuclear genome [3–5]. However, nuclear genome mutations have a rather low diagnostic and prognostic significance compared to certain traditional risk factors of atherosclerosis and cardiovascular diseases. A relative risk of each known polymorphism associated with atherosclerosis or CVD is 1.06–1.40. A total risk of cardiovascular disease presence for known nuclear genome polymorphisms is approximately 5% [6].

According to literature data, various pathologies are associated with certain mitochondrial mutations [7–9]. Mitochondrial genome mutations correlate with different diseases such as diabetes, some forms of deafness, myopathy, coronary vessel stenosis, predisposition to acute myocardial infarction, and cardiomyopathy. These pathologies often occur together with atherosclerosis [7–9].

Investigations of researchers all over the world are mainly dedicated to autosomal mutations, associated with atherosclerosis [10–12]. Only several works are dedicated to molecular-genetic defects of mitochondrial genome associated with atherosclerotic lesions [13–15]. In most of such investigations, large-scale deletions, leading to a full dysfunction of mitochondrial genome, were analyzed [16–18].

It is necessary to mention that because of mitochondrial genome instability, somatic mutations often occur in mitochondrial genome. While analyzing the association of mitochondrial genome with pathologies, it is necessary to carry out a quantitative assessment of mitochondrial genome heteroplasmy level [19–21].

In the present study, an association of eleven mitochondrial genome mutations with atherosclerosis was analyzed in individuals, aortic intima of which was morphologically divided into segments with atherosclerotic lesions of varying severity.

2. Materials and Methods

2.1. Materials. 265 normal and atherosclerotic segments of morphologically mapped aortic walls of varying severity of five individuals were taken as a material for investigation.

Samples of autopsy material were taken from thoracic section of aortic intima of men and women, who died at the age of 30–65 years as a result of an accident or a sudden death (except acute alcoholic and other intoxications and electrical injury).

To determine a type of a lesion, normal segments of aortas and segments with atherosclerotic lesions were identified macroscopically and then microscopically in accordance with classification of Atherosclerosis Board of American Heart Association [2].

Exteriorly unchanged segments of aorta had a smooth luminal surface. On vertical section in intima, it was possible to detect 2 layers: adjacent to aorta opening proteoglycan layer and an adjoining media myoelastic layer.

Segments of intima with primary lesions (lesion type I) were macroscopically segments with smooth yellowish surface, sometimes with small yellow spots. Microscopical changes were minimal. Small accumulations of extracellular lipidic drops in connective tissue matrix were observed. Along with tissue-fixed cells, in segments of preliminary lesions, there were more mononuclear cells compared to normal intima. There were no changes in tissue structure detected.

Fatty streaks (lesion type II) macroscopically were stripes and spots of yellow colour, slightly standing out over the vessel surface. Fatty streaks often merge together forming larger structures (clusters). In tissue sections, lipids were detected mainly intracellularly. Intracellular lipids were also detected in connective tissue matrix. Sometimes there can be observed an excessive overgrowth of extracellular matrix in fatty streaks.

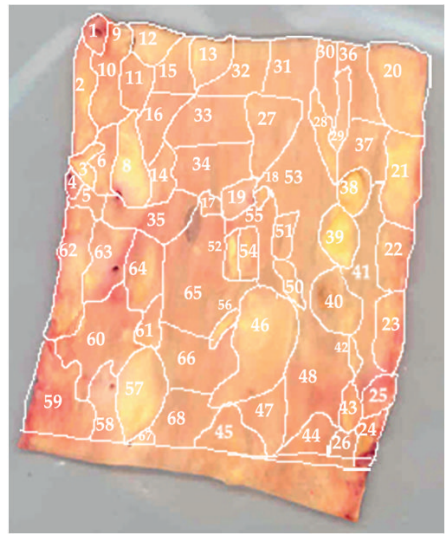

Figure 1: Morphological map of aorta number 1.

During macroscopic investigation, lipofibrous plaques (lesion type Va) looked like yellowish or pearl round or ellipsoidal formations standing far out over luminal surface. Microscopically in these lesions, all the changes were detected, which are characteristic for fatty streaks: accumulation of intracellular lipids and overgrowth of extracellular matrix. Furthermore, there were a massive necrotic nucleus and a connective tissue cap found in lipofibrous plaques. In lipofibrous plaques, there were such segments, which are morphologically similar to segments of lipidic lesions, arms of lipofibrous plaques.

Fibrous plaques (lesion type Vc) are macroscopically highly elevated round and oval formations of pearl colour, microscopically consisting mainly of coarse connective tissue matrix with cells "mured" in the matrix.

Aortic intima was morphologically mapped according to the presence of atherosclerotic lesions of varying severity or healthy vascular tissue.

Morphological Mapping of Aortas. In this study, 5 aortic samples were used. Each sample was a segment of a vascular wall with a size approximately $7 \times 9\ \text{cm}^2$, divided according to morphological characteristics into regions with atherosclerotic lesions of varying severity (1—normal tissue, 2—fatty infiltration, 3—fatty streak, 4—lipofibrous plaque, 5—fibrous plaque). In each aortic sample, 38 to 70 of such regions were identified (Figures 1, 2, 3, 4, and 5). In total, 265 segments of aortic intima were analyzed (Table 1).

Morphological maps for each aortic sample were made and in each morphological map the relative positions of the identified regions were marked. Morphological maps of investigated aortas are presented on Figures 1–5.

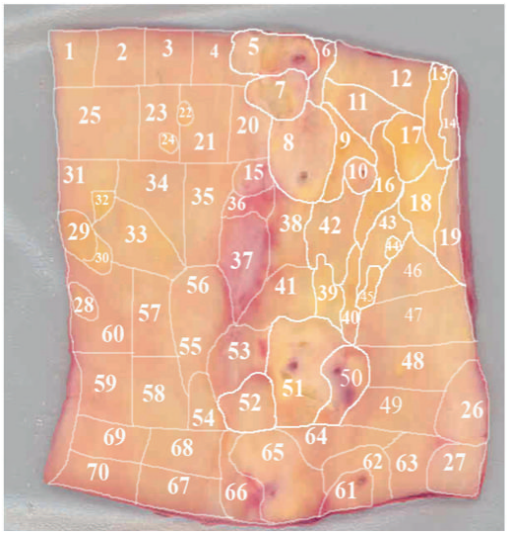

Figure 2: Morphological map of aorta number 2.

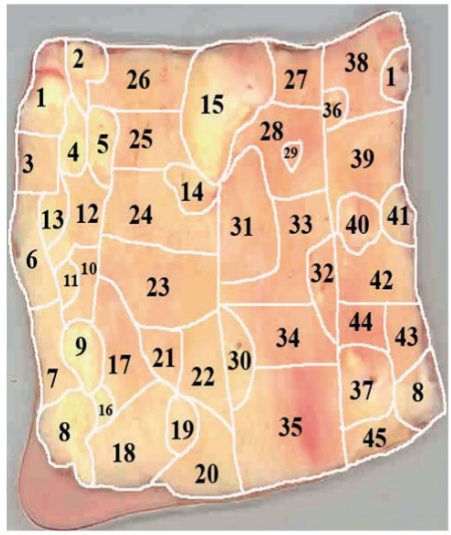

Figure 4: Morphological map of aorta number 4.

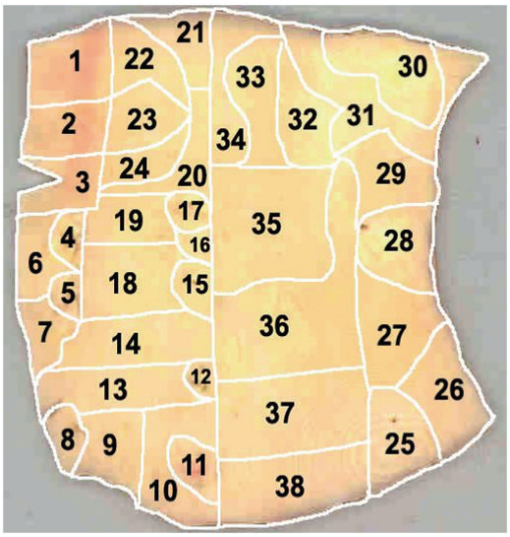

Figure 3: Morphological map of aorta number 3.

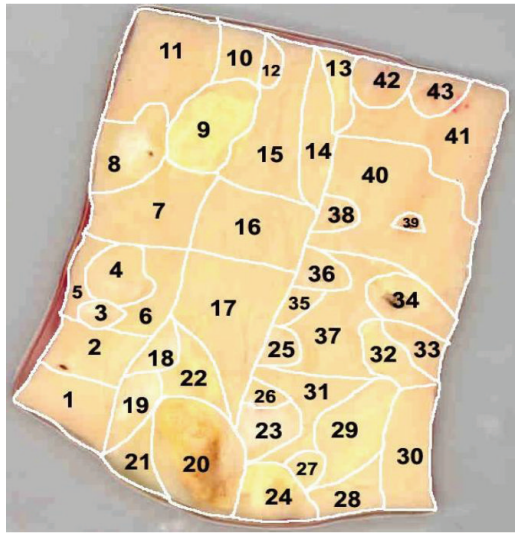

Figure 5: Morphological map of aorta number 5.

Furthermore, an opportunity of making a broader classification of regions was taken: normal tissue, early lesions (combination 2+3), and late lesions (combination 4+5).

2.2. Methods

2.2.1. DNA Isolation. Isolation of total DNA from aortic tissue samples (10 μg) was carried out using the phenol-chloroform extraction with the proteinase K lysis. The concentration of the DNA solution was measured by nanospectrophotometer IMPLEN NanoPhotometer at a wavelength of 260 nm.

2.2.2. PCR. For PCR, we used DNA with concentration 0.1 μg/mL and primers with concentration 10 pmol/μL. Sequences of primers for PCR are presented in Supplemental Table 1 (see Supplemental Table 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2014/825468).

A reaction mixture and PCR conditions were as follows:

MQ (H_2O)-4.6 μL;

dNTPs mixture 10X: 2 mM dATP, 2 mM dTTP, 2 mM dGTP, and 2 mM dCTP-4 μL;

10X buffer (16.6 μM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8)-4 μL);

$MgCl_2$: 25 mM

(i) 4 μL (the required concentration is 2.5 mM);
(ii) 2.4 μL (the required concentration is 1.5 mM);

Taq- polymerase ("Syntol," Russia)-1.33 μL

DNA template-4 μL;

Table 1: Atherosclerotic lesion degree of morphologically mapped aortas.

Number of aorta	Lesion degree	Quantity of segments
1	Normal tissue	9
	Fatty infiltration	17
	Fatty streak	6
	Lipofibrous plaque	5
	Fibrous plaque	1
2	Normal tissue	13
	Fatty infiltration	10
	Fatty streak	7
	Lipofibrous plaque	12
	Fibrous plaque	3
3	Normal tissue	12
	Fatty infiltration	6
	Fatty streak	6
	Lipofibrous plaque	12
	Fibrous plaque	7
4	Normal tissue	15
	Fatty infiltration	14
	Fatty streak	18
	Lipofibrous plaque	12
	Fibrous plaque	9
5	Normal tissue	25
	Fatty infiltration	17
	Fatty streak	13
	Lipofibrous plaque	10
	Fibrous plaque	5

primer F (+)-2.7 μL;

primer R (−)-2.7 μL.

The reaction was carried out in 40 μL volume.

Conditions for PCR are presented in Supplemental Table 2.

2.2.3. Electrophoresis of DNA Samples and PCR-Fragments. An electrophoresis of extracted DNA samples and PCR-fragments was performed in horizontal apparatus ("Helicon," Russia) in agarose gel with the use of 0.5XTBE buffer. The concentration of agarose (≪Fluka≫) was 0.8% (for DNA samples) and 1.5–2.0% (for PCR-fragments).

One of the primers was biotinylated with the aim of further pyrosequencing of PCR-fragment. The investigation was performed with the use of an amplifier ≪PTC DNA Engine 200≫.

The examples of gel electrophoresis of PCR-fragments of investigated mitochondrial mutations are presented in Supplemental Figures 1–4.

2.2.4. Pyrosequencing. After carrying out PCR, the PCR-fragments were pyrosequenced to detect point substitutions, microinsertions, or microdeletions of human mitochondrial genome. The investigation was performed with an automatic pyrosequencing system PSQ HS96MA. During the experiment, a scheme of sample conditioning, described in a manual attached to the pyrosequenator, was realized (sepharose particles were used). Sequences of primers for sequencing are listed in Supplemental Table 3.

A visualization of results was performed using a software attached to the pyrosequencing system. The statistical evaluation of the results was performed by using SPSS version 21.0.

Table 2: Bootstrap analysis of a correlation coefficient between a heteroplasmy level and the presence of a total atherosclerotic lesion of mapped aortas.

Mutation	Correlation coefficient value	Asymptotical significance (2-tailed)
652delG	0.311*	0.088*
652insG	−0.301	0.121
A1555G	−0.307	0.113
C3256T	0.353**	0.050**
T3336C	0.439**	0.023**
C5178A	0.357**	0.047**
G12315A	0.403**	0.045**
G13513A	−0.456**	0.035**
G14459A	0.453**	0.036**
G14846A	−0.297	0.129
G15059A	0.451**	0.037**

*Correlation of mutations with atherosclerotic lesions at $P \leq 0.1$ level of significance; **significant correlation of mutations with atherosclerotic lesions ($P \leq 0.05$).

3. Results

For assessment of 11 mitochondrial mutations, identified as potential atherosclerosis markers, an investigation on autopsy material with morphological and mutational mapping was made.

3.1. Analysis of 11 Mitochondrial Genome Mutations. During the analysis of all the segments of normal and atherosclerotic intima of 5 aortas by means of bootstrap analysis, it was found that total atherosclerotic lesion of mapped aortas is positively associated with mitochondrial genome mutations C3256T, T3336C, C5178A, G12315A, G14459A, and G15059A (significantly) and mutation 652delG is at $P \leq 0.1$ level of significance. Meanwhile, mutation G13513A had a significantly negative association with total atherosclerotic lesion of mapped aortas (Table 2).

According to Wilcoxon Matched-Pairs Signed-Ranks test (on averaged data, it is for all the aortas simultaneously), it was found (Table 3) that mitochondrial genome mutations, characteristic for total atherosclerotic lesion, are associated at the same level of significance with primary total atherosclerotic lesion (lipidic spots and fatty streaks) and a total of all the segments of lipofibrous plaques. Mutation G13513A

Table 3: Major mitochondrial genome mutations in different types of total atherosclerotic lesion of morphologically mapped aortas.

Mutations	Primary total atherosclerotic lesion		Lipofibrous plaques		Fibrous plaques	
	Correlation coefficient	Asymptomatic significance	Correlation coefficient	Asymptomatic significance	Correlation coefficient	Asymptomatic significance
652delG	0.093	0.146	0.308*	0.091*	0.323*	0.071*
652insG	−0.075	0.186	−0.095	0.125	−0.058	0.232
A1555G	−0.359**	0.048**	−0.401**	0.039**	−0.084	0.195
C3256T	0.368**	0.045**	0.407**	0.045**	0.352**	0.050**
T3336C	0.426**	0.034**	0.437**	0.025**	0.103	0.119
C5178A	0.365**	0.046**	0.439**	0.023**	0.356**	0.048**
G12315A	0.353**	0.050**	0.409**	0.041**	−0.367**	0.046**
G13513A	−0.423**	0.035**	−0.437**	0.021**	0.095	0.143
G14459A	0.403**	0.042**	0.463**	0.026**	0.073	0.191
G14846A	−0.107	0.117	−0.351**	0.050**	0.052	0.214
G15059A	0.405**	0.043**	0.471**	0.015**	0.062	0.203

*Correlation of mutations with atherosclerotic lesions at $P \leq 0.1$ level of significance; **significant correlation of mutations with atherosclerotic lesions ($P \leq 0.05$).

correlates with these types of total lesion significantly negatively. Moreover, mutation A1555G negatively correlates with primary total atherosclerotic lesion and a total of segments of lipofibrous plaques at $P \leq 0.05$ level of significance. During analyzing a total of segments of lipofibrous plaques, a significantly negative correlation of mutation G14846A with this type of lesion was found.

At the same time, there was found a positive correlation between a total of segments of lipofibrous and fibrous plaques and mitochondrial genome mutation 652delG at $P \leq 0.1$ level of significance. However, this mutation is absent in primary total atherosclerotic lesion.

Moreover, a total of fibrous plaque segments correlated at $P \leq 0.05$ level of significance with mutations C3256T and C5178A (positively) and G12315A (negatively).

3.2. Cumulative Mutational Burden of 11 Mutations in Morphologically Mapped Aortas. To detect the presence of mutational burden interrelation with the degree of atherosclerotic lesion, a linear regression analysis was carried out. Because of a high individual variability of the characteristic, these values were normalized as quartiles. In every aorta for each mutation, an investigation of heteroplasmy index distribution was carried out and interquartile boundaries were detected. Individual scalar indexes of heteroplasmy were transformed into single values 1, 2, 3, or 4, characterizing a belonging of an index to a certain quartile within the limits of a given autopsy sample. The results are presented in Tables 4 and 5.

Therefore, during the assessment of cumulative burden for 11 mutations, the model of linear regression reached $P < 0.001$ level of significance. Taking into consideration a sufficient quantity of degrees of freedom (11), it can therefore be said that the degree of atherosclerotic lesion is associated with cumulative burden for these mutations with 99.9% probability of error-free prognosis.

For each mutation associated with atherosclerosis, sensitivity and specificity indexes were studied. The analysis was carried out by a method of ROC-curve constructing with a further evaluation of the area under the curve. This enabled us to describe explanatory characteristics of genotypic markers (Figure 6).

Table 4: Summary of a linear regression model of mutational burden with a degree of atherosclerosis in aortas.

Model	R	R^2	Corrected R^2	Standard error of estimation
1	0.945	0.894	0.886	0.278

Note that predictors of the model were a constant, quartiles of G14846A, quartiles of 625delG, quartiles of T3336C, quartiles of C5178A, quartiles of A1555G, quartiles of G14459A, quartiles of G15059A, quartiles of 625insG, quartiles of G12315A, quartiles of G13513A, and quartiles of C3256T.

For this analysis, rank values (quartile numbers) of heteroplasmy were summed according to a sign of beta coefficient, obtained during regression analysis (if the value of a coefficient was positive, addition was done; if the value was negative, subtraction was done). The obtained parameter was named "mutational burden."

During the model using, the sensitivity index was 88.2 ($P \leq 0.05$, because a 95% confidence interval lies in the range from 74.6 to 95.3). Specificity index was 77.1 ($P \leq 0.05$, because a 95% confidence interval lies in the range from 70.8 to 87.3).

Therefore, cumulative mutational burden for 11 investigated mitochondrial genome mutations is associated with 88.2% of cases of atherosclerotic lesions in morphologically mapped aortas.

4. Discussion

Blood cells play a great role in the origin and development of atherosclerotic lesions in arterial intima. In atherogenesis, they migrate through endothelium and intima-medial layer of vessels. Meanwhile, lymphocytes play a signaling

Table 5: Dispersion analysis of linear regression model of mutational burden with a degree of atherosclerosis.

Model		Sum of squares	Degrees of freedom	Mean square	F	Significance
1	Regression	93.8	11	8.53	110.0	<0.001
	Residual	11.2	144	0.08		
	Total	**104.9**	**155**			

Note that predictors of the model were a constant, quartiles of G14846A, quartiles of 625delG, quartiles of T3336C, quartiles of C5178A, quartiles of A1555G, quartiles of G14459A, quartiles of G15059A, quartiles of 625insG, quartiles of G12315A, quartiles of G13513A, and quartiles of C3256T.

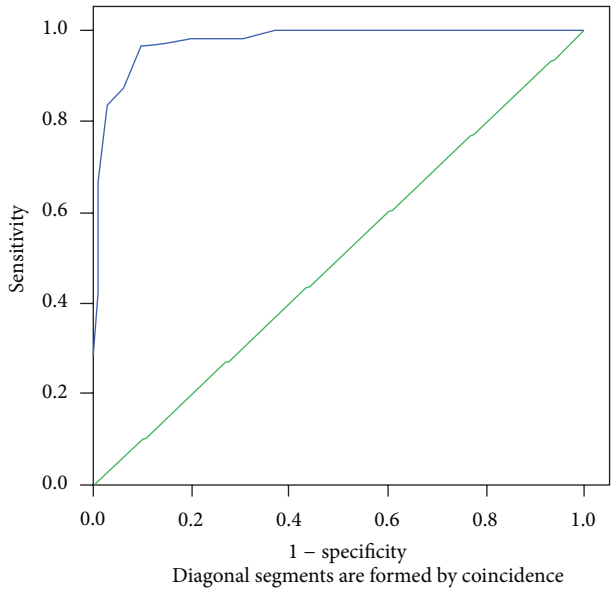

Figure 6: ROC-curve for assessment of sensitivity and specificity of an index "mutational burden" concerning atherosclerosis. Positive real state is an atherosclerotic plaque. The area under the curve is 0.975 ($P < 0.001$).

role in immune and inflammatory response formation and monocytes form macrophage cells, aimed at removing the abundance of cholesterol, accumulated in the atherosclerotic lesion focus. A probable role of mitochondrial genome mutations in the origin and development of atherosclerosis may be the fact that these mutations result in protein chain defects in respiratory chain enzymes of mitochondria or transfer RNA. Meanwhile, a metabolism level of defective mitochondria lowers and as a result monocytes containing them acquire a liability to lipoidosis. Therefore, a result of pathophysiologic processes, started by mitochondrial genome mutations, is a transformation of mutant monocytes into foam cells.

To test this hypothesis, a decision to carry out a comparative analysis of heteroplasmy level in normal and affected by atherosclerosis human arterial intima was made.

To detect a heteroplasmy level in the samples investigated, a new original method of quantitative assessment of the mutant allele of mitochondrial genome [22, 23], based on pyrosequencing technology, was developed by the authors [24]. This method is suitable for the study of any biological samples. With its help, it is possible to detect the heteroplasmy level of both hereditary and somatic mitochondrial genome mutations which appear during the life of an individual or in pathological processes. Furthermore, it was possible to detect the percentage of nuclear genome somatic mutations, occurring, for example, in the course of occurrence and development of cancer processes.

In the present study, for the first time we obtained data, demonstrating that different segments of the aortic intima, both normal and having atherosclerotic lesions of varying severity, may differ in heteroplasmy level of mutant allele of mitochondrial genome. According to the literature, the investigated mutations are associated with different pathologies (Table 6).

Most types of aortic atherosclerotic lesions turned out to be associated with mutations C3256T, T3336C, C5178A, G12315A, G14459A, and G15059A, which may indicate that the key point in the start of pathophysiological mechanisms, which result in atherosclerotic lesions formation in human aortas, is defects in transport RNA-Leu (codons recognized UUR and CUN) and also 1, 2, and 6, NADH-dehydrogenase subunit, and cytochrome B.

Conspicuous is the fact that the mutation spectrum in different types of atherosclerotic lesions of the aortic intima may slightly differ. For example, mutation A1555G showed an antiatherogenic effect in early atherosclerotic lesions (lipidic spots and fatty streaks) and lipofibrous plaques. At the same time, antiatherogenic mutation G14846A turned out to be typical only for lipofibrous plaques.

In fibrous plaques, mutation spectrum significantly differed from other types of atherosclerotic lesions. Proatherogenic mutations were C5178A and C3256T; antiatherogenic mutation was G12315A. Apparently, the defective parts in G12315A transport RNA-Leu (codon recognized CUN) play an important role in pathophysiological process of the origin and development of atherosclerotic lesions before the fibrous plaque stage begins, when the percentage of heteroplasmy for this mutation decreases to such an extent that it is higher in normal intima than in the type of atherosclerotic lesion. The heteroplasmy level of some other mitochondrial genome mutations in fibrous plaques apparently decreases and does not vary from this parameter in normal aortic intima.

5. Conclusion

In the present study, a focality of atherosclerotic lesions in human aortic intima was confirmed. The basis for this confirmation was differences in the heteroplasmy level of 11 mitochondrial mutations for different segments of both

Table 6: Data on the pathologies, caused by the investigated mutations.

Gene	Mutation	Pathology
Gene 12S rRNA	652insG	Gastric carcinoma [25]
	A1555G	Dullness of hearing, induced by aminoglycosides and idiopathic hearing loss, sensibility to aminoglycoside antibiotics; deafness [26–28]
Gene tRNA-Leu (codon recognized UUR)	C3256T	MELAS, encephalopathy, lactic acidosis, myopathy, cardiomyopathy, stroke-like lesion in the right parietooccipital brain region, and oxidative defect of muscular metabolism [8]
Gene of subunit 1 NADH dehydrogenase	T3336C, a silencing mutation	Type 2 diabetes mellitus [29]
Gene of subunit 2 NADH dehydrogenase	C5178A causes a substitution of leucine for methionine	Acute myocardial infarction [30]
Gene tRNA-Leu (codon recognized CUN)	G12315A	Encephalopathy [31]
Gene of subunit 5 NADH dehydrogenase	G13513A	Li syndrome (hereditary encephalomyopathy), Wolff-Parkinson-White syndrome (preexcitation syndrome), and cardiomyopathy [7, 32]
Gene of subunit 6 NADH dehydrogenase	G14459A (a substitution of alanine for valine in the seventy-second amino acid position, which is located in the most conservative region of protein ND6)	Hereditary Leber's optic atrophy. Associated with dysfunction of basal ganglia, muscular spasticity, and encephalopathy [33, 34]
Gene of cytochrome B	G14846A (a substitution of glycine for serine in position 34 (G34S) that weakens enzymatic function of cytochrome B)	Mitochondrial myopathies [35]
	G15059A (a nonsense mutation, as the result of which amino acid glycine in position 190 is substituted for a terminating codon, causing a stopping in translation, protein size reduction, and a loss of 244 amino acids from C-terminus of a protein). The mutation weakens enzyme function of cytochrome B.	Mitochondrial myopathies [35]

normal and atherosclerotic morphologically mapped human aortic intima. It was found that a certain spectrum of pro- and antiatherogenic mitochondrial genome mutations is characteristic for different types of atherosclerotic lesions of aortic intima. In total atherosclerotic lesion, lipofibrous plaques, and primary atherosclerotic lesions of the aortic intima, six proatherogenic mutations (C3256T, T3336C, C5178A, G12315A, G14459A, and G15059A) and one antiatherogenic mutation (G13513A) were identified. Differences in the spectrum of mutations between primary atherosclerotic lesions, lipofibrous plaques, and total atherosclerotic lesions lie in the fact that, for the first two types, antiatherogenic mutation A1555G is representative. In lipofibrous plaques, mutation G14846A is also antiatherogenic. The mutation spectrum in fibrous plaques is different from other types of atherosclerotic lesions. Proatherogenic mutations in fibrous plaques are C5178A and C3256T. Mutation G12315A is antiatherogenic.

The findings of this study may be useful for practitioners and medical geneticists for early detection and family analysis of atherosclerosis.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

This study was supported by the Russian Scientific Foundation, Grant no. 14-14-01038.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 490295, 9 pages
http://dx.doi.org/10.1155/2015/490295

Research Article

TRAF1/C5 but Not *PTPRC* Variants Are Potential Predictors of Rheumatoid Arthritis Response to Anti-Tumor Necrosis Factor Therapy

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Received 25 July 2014; Accepted 26 November 2014

Academic Editor: María E. Esteban

Background. The aim of our work was to replicate, in a Southern European population, the association reported in Northern populations between *PTPRC* locus and response to anti-tumor necrosis factor (anti-TNF) treatment in rheumatoid arthritis (RA). We also looked at associations between five RA risk alleles and treatment response. *Methods*. We evaluated associations between anti-TNF treatment responses assessed by DAS28 change and by EULAR response at six months in 383 Portuguese patients. Univariate and multivariate linear and logistic regression analyses were performed. In a second step to confirm our findings, we

pooled our population with 265 Spanish patients. *Results*. No association was found between *PTPRC* rs10919563 allele and anti-TNF treatment response, neither in Portuguese modeling for several clinical variables nor in the overall population combining Portuguese and Spanish patients. The minor allele for RA susceptibility, rs3761847 SNP in *TRAF1/C5* region, was associated with a poor response in linear and logistic univariate and multivariate regression analyses. No association was observed with the other allellic variants. Results were confirmed in the pooled analysis. *Conclusion*. This study did not replicate the association between *PTPRC* and the response to anti-TNF treatment in our Southern European population. We found that *TRAF1/C5* risk RA variants potentially influence anti-TNF treatment response.

1. Introduction

Rheumatoid arthritis (RA) is an inflammatory, chronic, and disabling disease. Methotrexate is the most widely used disease modifying antirheumatic drug (DMARD) in RA treatment. However, for refractory and severe cases, anti-tumor necrosis factor (anti-TNF) therapy has become a cornerstone of RA treatment strategy [1]. These drugs have revolutionized RA treatment and prognosis in the last 10–15 years. Nevertheless, only approximately one-third of patients achieve remission and the other third will eventually fail to respond [2]. In a multifactorial and polygenic disease like RA, it is expected that response to treatment may be influenced by genetic, clinical, and biological factors [3]. The identification of predictors of response is of crucial importance to optimize the cost-effective use of expensive medications, such as anti-TNF therapy. Large registries collecting information on sociodemographic characteristics, disease activity, functional status, and treatments have allowed the study of clinical predictors of response [4–7].

Genetic variants associated with RA susceptibility include the *HLA-DRB1* region containing shared epitope alleles (SE), which is also associated with severity [8, 9]. Outside the major histocompatibility complex (MHC), *PTPN22*, *TRAF1/C5*, and *TNFAIP3* loci were the most consistently associated with susceptibility and *TRAF1/C5* region also with RA severity [10, 11] and noncardiovascular mortality in some populations [12].

In the nineties, studies performed to look at associations between treatment response and the presence of SE indicated that the response to disease modifying antirheumatic drugs (DMARDs) such as methotrexate (MTX), in combination or monotherapy [13], and more recently with leflunomide [14], etanercept [15], and infliximab [16], was better in the presence of SE.

In recent years, several studies of potential associations between anti-TNF treatment response and polymorphisms in the promoter region of the *TNF* gene (positions −308 and −238), and other related genes such as *lymphotoxin-α* and *TNF receptors*, showed contradictory results [17–20]. With the increasing knowledge on RA pathophysiology and genomewide studies demonstrating that loci related with TNF signaling pathways such as the NF-kB signaling pathway (*TRAF1/C5*, *TNFAIP3*, and *REL*) and other pathological processes such as enhanced citrullination (*PADI4*) may increase RA risk, it is compelling to explore how those loci could also influence the anti-TNF response [21–28].

Cui et al., analyzing thirty-one risk allele variants, found that the major allele (G) of the rs10919563 *PTPRC* locus, which is a known predictor of RA risk, was associated with an increased response to anti-TNF therapy, with stronger association in seropositive patients (either anticitrullinated peptides antibodies, ACPA, and/or rheumatoid factors (RF)) [29]. The authors did not find any association with treatment response among the other thirty RA-associated risk alleles studied. In that multicohort study, potential associations between response and *HLA-DRB1* were not assessed. One study from BRAGGSS, UK, showed no association between *HLA-DRB1* and *PTPN22* variants and response to anti-TNF treatment [30]. However, another study from the UK confirmed the association between *PTPRC* variants and response in the entire cohort, reporting no significance in the ACPA positive group alone [31].

The challenge over the next years will be to identify the RA stages in which genetic variants exert their maximum influence and also to unveil their clinical significance and usefulness as potential therapeutic targets or biomarkers [10].

In this study we aimed to replicate in a Southern European population the association between rs10919563 *PTPRC* variants and the response to anti-TNF treatment found in previous studies. We also aimed to test whether *HLA-DRB1* and other five selected RA susceptibility genes may influence the response to anti-TNF treatment—that is, potential associations between anti-TNF treatment response and risk RA loci related with NF-kB signaling pathway (*TRAF1/C5*, *TNFAIP3,* and *REL*), citrullination (*PADI4*), and the genetic variants inside the MHC (*HLA-DRB1* 04* high-resolution (4-digit) genotyping) and outside the MHC (*PTPN22* locus) with the strongest association with RA risk. The analyses were modeled adjusting for clinical variables that influenced treatment response.

2. Material and Methods

2.1. Patients. Primary analyses were performed upon Reuma.pt, the National Register for Rheumatic Diseases from the Portuguese Society of Rheumatology (SPR) established in 2008, which captures more than 90% of patients treated with biological therapies managed in rheumatology departments across Portugal [32]. The register is linked to the Biobanco-IMM [33]. Blood samples were collected from November 2010 up to May 2011 at six major centers. Information on disease activity and treatments has been collected by rheumatologists at every infusion for intravenous drugs and every 3 months for subcutaneous biologic therapies. The decision to initiate and maintain the treatment was guided by the SPR's recommendations [34]. RA patients fulfilling the American College of Rheumatology

(ACR) 1987 revised criteria [35] were eligible for this study whether they were treated with an anti-TNF agent as the first biologic therapy, had a follow-up of at least six months, or had a blood sample collected for DNA assessment. Patients with self-reported non-Caucasian ancestry and those with missing values for DAS28 at baseline or at six months were excluded. Reuma.pt was approved by National Board of Data Protection and Health National Directorate.

In a second step for confirming our findings, we pooled our Portuguese sample with Spanish RA patients from the Rheumatology Department of Virgen de las Nieves (Granada, Spain) and Reina Sofia (Córdoba, Spain) Hospitals, selected with the criteria described above.

The study was conducted in accordance with the regulations governing clinical trials such as the Declaration of Helsinki and was approved by the Hospitals' Ethics Committees. Patients signed an informed consent for research use of their clinical data and blood samples.

2.2. DNA Extraction and Genotyping. DNA was extracted from whole blood using the QIAamp DNA Blood Mini Kit combined with the automated extraction device QIAcube (QIAGEN, Hilden, Germany), according to the manufacturer's instructions.

[*PTPRC*] rs10919563 (A/G) was previously reported in association with anti-TNF treatment response [29, 31]. The other five SNP markers were selected based on (1) relevance for RA biologic NF-kB pathway, [*6q23-TNFAIP3*] rs10499194 (C/T), [*REL*] rs13031237 (G/T), and [*TRAF1/C5*] rs3761847 (A/G); (2) role on citrullination process, [*PADI4*] rs2240340 (C/T); and (3) strong association with RA risk, [*PTPN22*] rs2476601 (A/G). *HLA-DRB1* 04* high-resolution genotyping was performed by PCR sequence-specific primers (SSP) using Olerup SSP DRB1*04 typing Kit (Olerup SSP AB) as described in the manufacturers' protocols.

Samples were run with Luminex xMAP system (Tepnel Lifecodes). Allele call was obtained with Quicktype for Lifematch 2.6 software. The samples were genotyped using Taqman SNP genotyping assays (Applied Biosystems, Foster City, USA) as described in the manufacturers' protocols.

For purposes of quality control, 95% of sample threshold and 95% genotyping success threshold were used. Exclusion criteria also included a minor allele frequency <0.1 and deviation from Hardy-Weinberg equilibrium. In the end, *HLA-DRB1* 04* and six allele variants were analyzed.

2.3. Statistical Analysis

2.3.1. Outcome Measures and Covariates. Our primary outcome was the change in disease activity score in 28 joints including erythrocyte sedimentation rate measures (DAS28ESR) between the drug start date and six months of treatment (in our study, a positive variation means a decrease in disease activity at six months in comparison with baseline visit) [36]. Secondary outcome was the proportion of nonresponders versus good responders (excluding moderate responders) defined by the EULAR response criteria at six months [37]. Nonresponse was defined by an absolute change in DAS28 $\leq$ 0.6 or a change in DAS28 between 0.6 and 1.2 with a DAS28 at six months >5.1. Good response was defined as change in DAS28 of >1.2 and DAS28 at six months $\leq$3.2. In this study, nonresponse was the reference category, with logistic regression analysis modeling the probability of achieving good response. For both outcomes, covariates' coefficient > 0 or odds ratio (OR) > 1 predicted favorable response.

The predictors of interest were the minor allele variants of *PTPRC* and the five SNPs described above as well as the presence of SE (*DRB1* 0401/04/05/08* and *DRB1* 1001*), as previously described in the RA Portuguese population [8].

Covariates collected at drug start date (baseline visit) were gender, age, age at diagnosis, disease duration, years of education, smoking (ever/never), RF (positive/negative), ACPA (CCP2, positive/negative), extra-articular manifestations (yes/no), concomitant therapy with corticosteroids (yes/no), any disease modifying antirheumatic drugs (DMARDs) including MTX (yes/no), DAS28, Health Assessment Questionnaire (HAQ), and physician's global assessment of disease activity (PhGA). We tested the association between these variables and change in DAS28 at six months by univariate analyses. Then, we built a multivariate linear model with the significant baseline clinical covariates (at a $P < 0.05$). The variables that remained significant after adjustment entered the multivariate model for each SNP.

In a second step, in order to confirm our results, we pooled the Spanish population and looked at associations between the SNPs and treatment response.

2.3.2. Primary Analysis. Each SNP was tested for an association with the anti-TNF response, taking anti-TNF drugs as a group. Univariate linear regression analyses for the primary outcome and logistic regression for the secondary outcome were performed, using additive models. Homozygotes for major alleles were classified as 0, 1 for heterozygotes, and 2 for minor allele homozygotes. The response was also modeled with multivariate models including significant baseline clinical predictors for treatment response.

2.3.3. Secondary Analyses. For the loci that presented a significant association with treatment response in the primary analysis, we tested the same relationship by stratifying the patients in ACPA positive and ACPA negative groups.

The study had >80% power to detect a change of >0.6 in the DAS28 score (considered a clinically meaningful change [37]) for allele frequencies > 0.1 and a type I error of <0.05.

There were no assumptions about the direction of effect on treatment response. Results were considered significant for a two sided P value <0.05. Analyses were performed using SAS version 9.3 (SAS Institute, Cary, NC, USA).

3. Results

PTPRC, additional five SNP markers, and *HLA-DRB1* genotyping were assessed in 416 Portuguese patients. Twenty-seven reported a non-Caucasian ancestry and were excluded. Six were excluded for absence of DAS28 at baseline or at six

months, leaving 383 patients for analysis. Table 1 describes the baseline demographic and clinical characteristics for the 383 RA Portuguese patients included in the study.

At six months, 119 (31.1%) patients were classified as good responders, 175 (45.7%) as moderate responders, and 89 (23.2%) as nonresponders according to the EULAR response criteria [37].

Number of years completed at school, HAQ, and DAS28 at baseline were found to have a significant association with treatment response at six months and were included in the multivariate models (Table 2).

We did not find association between *PTPRC* rs10919563 and anti-TNF treatment response (Table 3).

Univariate analysis looking for association between the other five risk alleles and anti-TNF treatment response demonstrated a worse response for minor (G) allele, rs3761847 SNP, in *TRAF1/C5* region either measured by a change in DAS28 at six months (coefficient (coef.) −0.24; 95% confidence interval (CI) −0.43, −0.06; *P* value of 0.009) or by the proportion of good responders versus nonresponders at six months (OR 0.61; CI 0.41, 0.92; *P* value of 0.018). After adjusting for years of school completed, HAQ, and DAS28 at baseline, in two multivariate models, this association remained significant for a P value < 0.05 (Table 4). In the univariate linear model, rs3761847 accounted for 1.75% of the change in DAS28 at six months. The multivariate model with the clinical covariates described above explained 23.9% of this change. When rs3761847 was added to the clinical model, the R^2 increased to 25.4%.

There were no significant relationships between the other SNP markers tested and response to therapy (Table 3). Similarly, SE was not associated with response to anti-TNF therapy in our population (coef. 0.21, *P* value 0.21; OR 1.24, CI 0.56, 2.71).

In a secondary stratified analysis, the relationship between rs3761847 and treatment response in 278 ACPA positive and 105 ACPA negative patients was tested, but no association was detected (ACPA positive group, coef. −0.16, *P* value 0.09 and ACPA negative group, coef. −0.41, *P* value 0.04).

We replicated these findings pooling 265 Spanish RA patients and testing the association between the risk alleles and anti-TNF treatment response (results not shown).

Table 1: Baseline demographic and clinical characteristics of the 383 rheumatoid arthritis patients treated with anti-TNF drugs.

TNF inhibitor	
Adalimumab	79 (20.6)
Etanercept	139 (36.3)
Golimumab	10 (2.6)
Infliximab	155 (40.5)
Age (years)	52.5 (12.2)
Disease duration (years)	10.7 (8.9)
Female	343 (89.5)
Rheumatoid factor	290 (75.7)
ACPA	278 (72.6)
Extra-articular manifestations	91 (23.7)
Smoking-ever (n = 367)	71 (19.3)
Education (years)	7.1 (4.6)
DMARDs (n = 377)	346 (91.8)
MTX (n = 377)	310 (82.2)
Corticosteroids (n = 377)	277 (73.5)
DAS28 ESR	5.77 (1.1)
Physician global assessment (mm)	56.1 (16.5)
Health Assessment Questionnaire	1.45 (0.58)

Values shown are means (SD) or n (%).
TNF: tumor necrosis factor; ACPA: anticitrullinated peptides antibodies; DMARDs: disease modifying antirheumatic drugs; MTX: methotrexate; DAS: disease activity score; ESR: erythrocyte sedimentation rate.

Table 2: Multivariate model of baseline demographic and clinical variables as predictors of response to anti-TNF treatment at 6 months.

Baseline variables	Coefficient (*P* value)
Age (years)	−0.01 (0.25)
Disease duration (years)	0.01 (0.24)
Female gender	−0.01 (0.95)
ACPA (positive)	−0.07 (0.64)
Smoking (ever)	−0.06 (0.72)
Higher education (years)	0.03 (0.03)*
Corticosteroids (yes)	−0.16 (0.26)
DMARDs (yes)	0.05 (0.83)
Extra-artic. manif. (yes)	0.10 (0.49)
HAQ	−0.40 (0.002)*
PhGA (mm)	−0.001 (0.68)
DAS	0.60 (<0.001)*

* P value <0.05.
The probability of response to anti-tumor necrosis factor therapy at 6 months was modeled in a multivariate linear regression analysis.
The change in disease activity score assessing 28 joints between the baseline visit and the visit after 6 months of therapy was the continuous outcome.
ACPA: anticitrullinated peptides antibodies; DMARDs: disease modifying antirheumatic drugs; Extra-artic. manif.: extra-articular manifestations; HAQ: Health Assessment Questionnaire; PhGA: physician global assessment; DAS: disease activity score.

4. Discussion

Our study did not replicate the association previously published between *PTPRC* rs10919563 variant and the response to anti-TNF therapy in patients with RA. The analyses suggest that in our Southern European population, the minor (G) allele rs3761847 in the *TRAF1/C5* locus might have an association with poor response to anti-TNF treatment at six months. These results were consistent using either the absolute change in DAS28 or the proportion of good/nonresponders as outcomes in univariate and multivariate models adjusted for clinical predictors of response. The other four RA susceptibility loci tested and the *HLADRB1* were not associated with anti-TNF response.

In 2007, *TRAF1/C5* was identified as a risk locus for RA by Plenge and colleagues in a GWAS [21] and by Kurreeman et al. in a candidate gene approach [38]. More recently, it

Table 3: Minor allele genetic variants as predictors of response, analyzed by univariate and multivariate linear and logistic regression models.

SNP	Gene	Chr Position (bp)	Minor allele	MAF	Absolute change in DAS $n = 383$		EULAR good response versus nonresponse $n = 208$ (good responders = 119, nonresponders = 89)	
					Univariate additive linear model Coef (95% CI) P value	Multivariate additive linear model Coef (95% CI) P value	Univariate additive logistic model OR (95% CI) P value	Multivariate additive logistic model OR (95% CI) P value
rs2476601	*PTPN22*	1 114,179,091	A	0.11	0.28 (−0.01, 0.58) 0.058	0.12 (−0.15, 0.40) 0.38	1.68 (0.88, 3.21) 0.12	1.71 (0.82, 3.54) 0.15
rs2240340	*PADI4*	1 17,507,279	T	0.46	−0.11 (−0.29, 0.07) 0.22	−0.09 (−0.25, 0.08) 0.30	0.79 (0.54, 1.16) 0.22	0.81 (0.52, 1.24) 0.33
rs13031237	*REL*	2 60,989,633	T	0.45	−0.09 (−0.26, 0.09) 0.34	−0.14 (−0.31, 0.02) 0.09	0.91 (0.62, 1.33) 0.62	0.77 (0.50, 1.19) 0.24
rs10499194	*TNFAIP3*	6 138,002,637	T	0.29	−0.05 (−0.26, 0.15) 0.60	−0.06 (−0.26, 0.12) 0.50	0.79 (0.50, 1.25) 0.31	0.86 (0.52, 1.40) 0.55
rs10919563	*PTPRC*	1 196,967,065	A	0.11	−0.05 (−0.34, 0.23) 0.72	0.05 (−0.21, 0.32) 0.69	1.21 (0.65, 2.22) 0.55	1.61 (0.79, 3.26) 0.19

Values were significant for $P < 0.05$.

The analyses of response were modeled for the minor alleles. Additive models were used taking the homozygote for the major allele as the reference variable.

383 patients were included in the primary analysis (the outcome was the absolute change in the disease activity score (DAS28) between the baseline and the 6-month visit).

208 patients were assessed in the secondary analyses; 119 were good responders and 89 nonresponders according to the EULAR response criteria.

Values were presented as regression coefficient (coef.), 95% confidence intervals (CI), and P values (P) for linear regression analyses and as odds ratio (OR), 95% confidence intervals (CI), and P values (P) for logistic regression analyses.

In multivariate models, covariates included were number of years completed at school, health assessment questionnaire, and disease activity score at baseline.

SNP: single nucleotide polymorphism; Chr: chromosome; MAF: minor allele frequency.

Table 4: Association of the rs3761847 single nucleotide polymorphism of *TRAF1/C5* locus with the response to anti-TNF treatment.

SNP	Ch	Position Bp	Genotype	Count	MAF	Change in DAS	Absolute change in DAS $n = 383$ Linear regression models		EULAR good response versus nonresponse $n = 208$ (good = 119, non = 89) Logistic regression models	
							Univariate	Multivariate	Univariate	Multivariate
rs3761847	9	122,730,060	11	167	0.35	1.95 (1.26)	Coef. −0.24	Coef. −0.23	OR 0.61	OR 0.58
			12	165		1.82 (1.31)	CI −0.43, −0.06	CI −0.40, −0.06	CI 0.41, 0.92	CI 0.37, 0.91
			22	51		1.38 (1.19)	*P* 0.009	*P* 0.009	*P* 0.018	*P* 0.019

Values were significant for $P < 0.05$.

The analyses of response were modeled for the minor (G) allele, which is the risk allele for RA. Additive models were used with the homozygote for the two major alleles as the reference variable.

383 patients were included in the primary analysis (the outcome was the absolute change in the disease activity score (DAS28) between the baseline and the 6 month visit).

208 patients were assessed in the secondary analyses, 119 were good responders and 89 non-responders according to the EULAR response criteria.

Values were presented as regression coefficient (coef.), 95% confidence intervals (CI) and *P*-values (*P*) for linear regression analyses and as odds ratio (OR), 95% confidence intervals (CI) and *P*-values (*P*) for logistic regression analyses.

In multivariate models covariates included were number of years completed at school, health assessment questionnaire (HAQ) and disease activity score at baseline.

SNP: single nucleotide polymorphism; Ch: chromosome; Genotype 1 = major allele, 2 = minor allele; MAF: minor allele frequency; DAS: disease activity score.

was shown to be a marker of disease severity [10, 11] and in one study of noncardiovascular mortality [12]. No previous studies have reported the association with response to anti-TNF therapy. Due to a high level of linkage disequilibrium between the genes encoding TNF receptor associated factor 1 and complement component 5, it is currently not possible to assure which of these two genes at 9q33.2 encloses the causal variant. Both are possible candidates. The protein, TNF receptor-associated factor 1 (TRAF1), is a member of the TNF receptor (TNFR) associated factor (TRAF) protein family and is encoded by the *TRAF* gene. TRAF proteins associate with and mediate the signal transduction from various receptors of the TNFR superfamily. TRAF1 and TRAF2 form a heterodimeric complex, which is required for TNF mediated activation of MAPK8/JNK and NF-kB. The protein complex interacts with inhibitor-of-apoptosis proteins and mediates the antiapoptotic signals from TNF receptors. TRAF 1 is a negative regulator of TNF receptor and Toll-like receptor signaling and may contribute to the proliferation of T cells. rs3761847 is located at the upstream of *TRAF1* and the downstream of the complement fraction *C5* [39]. The clinical and biologic data for *C5* are equally relevant. The complement pathway has been implicated in the pathogenesis of rheumatoid arthritis for more than 30 years. C5 cleavage generates the proinflammatory anaphylatoxin C5a, as well as C5b, which initiates the generation of the membrane-attack complex. C5-deficient mice are resistant to inflammatory arthritis in models with a dominant humoral component [21, 40]. It is compelling to hypothesize that this variant or other causative variants at this locus may influence the function or expression levels of TRAF1 and/or C5, affecting RA susceptibility, severity, and anti-TNF treatment response. Functional studies are warranted to confirm these hypotheses. Nevertheless, our result could be that false positive and studies with other populations are required to confirm the replication of these findings.

We did not find any significant associations between response to treatment and the presence of SE, neither with the other four RA risk allele variants related with NF-kB signaling pathway nor with citrullination, chosen for its high association with RA susceptibility. We were also not able to replicate the previously reported *PTPRC* association with treatment response in our Southern European population. Plant et al. reported a P value of 0.04 for *PTPRC* association with anti-TNF treatment response and no significance for the ACPA positive patients in the stratified analysis. This result was strengthened with a meta-analysis combining their data with the Cui et al. study [29]. Although our sample size was large enough to detect allele variants association with response with a power > 80% for minor allele frequency (MAF) > 0.1, *PTPRC* showed a MAF of 0.11 which might have made difficult the detection of association. The different genetic background of our population could also account for the lack of replication. In a recent study, we and two Japanese groups also failed to replicate the results found in Northern European populations [41].

We are far from understanding the genetic mechanisms that underlie treatment response in patients with RA, as demonstrated by the large proportion of the variance explained by clinical factors compared to that explained by a single SNP. The ultimate goal for genetic, laboratory, and clinical predictors of treatment response studies is personalizing treatment and medicine practice by identifying biomarkers and specific phenotypes clinically useful for improving the therapeutic strategy. The identification of individual predictors may contribute to building complex algorithms aimed at improving the prediction of a better/worse response to anti-TNF drugs and other classes of biologic therapies.

5. Conclusions

We were not able to replicate the previously reported *PTPRC* rs10919563 association with treatment response in our Southern European RA population.

The minor (G) allele of rs3761847 in the *TRAF1/C5* locus, which is a susceptibility factor for RA related to TNF signaling, was associated with a poor response to anti-TNF treatment at six months, using either the absolute change in DAS28 or the proportion of good responders and non-responders as outcomes. This association was also observed after adjustment in a multivariate model with baseline clinical predictors of response.

We did not find any significant associations between response to treatment and the presence of SE, neither with the other four RA risk allele variants related with NF-kB signaling pathway nor with citrullination, chosen for its high association with susceptibility.

Additional studies in other populations are necessary to confirm the relevance of these findings.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This work was supported by a grant from Harvard-Portugal Program HMSP-ICS/SAU-ICT/0002/2010. Daniel H. Solomon received support for this work from the NIH (K24-AR-055989). Elizabeth W. Karlson received support for this work from NIH (K24-AR-AR0524). Reuma.pt received unrestricted grants from Abbott, Bristol-Myers Squibb, Merck Sharp and Dohme, Pfizer, Roche, and UCB Pharma.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 718786, 7 pages
http://dx.doi.org/10.1155/2015/718786

Research Article

Novel Mutations in the Transcriptional Activator Domain of the Human TBX20 in Patients with Atrial Septal Defect

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Received 15 August 2014; Accepted 20 October 2014

Academic Editor: María E. Esteban

Background. The relevance of *TBX20* gene in heart development has been demonstrated in many animal models, but there are few works that try to elucidate the effect of *TBX20* mutations in human congenital heart diseases. In these studies, all missense mutations associated with atrial septal defect (ASD) were found in the DNA-binding T-box domain, none in the transcriptional activator domain. *Methods*. We search for *TBX20* mutations in a group of patients with ASD or ventricular septal defect (VSD) using the High Resolution Melting (HRM) method and DNA sequencing. *Results*. We report three missense mutations (Y309D, T370O, and M395R) within the transcriptional activator domain of human TBX20 that were associated with ASD. *Conclusions*. This is the first association of TBX20 transcriptional activator domain missense mutations with ASD. These findings could have implications for diagnosis, genetic screening, and patient follow-up.

1. Introduction

Congenital heart defects (CHD) are the most common developmental defects in humans [1], affecting 6–8 out of 1,000 newborns [2–5]. In Mexico CHD have an estimated prevalence of 1% (10 out of 1,000 newborns) [6]. CHD are a group of multifactorial complex diseases with environmental and genetic factors playing important roles [7]. Mutations in genes, such as in the transcription factors genes *NKX2.5*, *TBX5,* and *GATA4*, have been correlated to the pathogenesis of CHD [8]. Although the research of the genes involved in cardiac developmental pathways is growing we are not able to completely define how mutations in these genes cause CHD.

The heart is formed in early stages of embryonic development. This process requires the action of several transcription factors that regulate through activation or repression of key genes in a specific temporary/spatial manner [7]. An important group of transcription factors involved in heart development is the T-box family. *T-box* genes mutations in humans are associated with CHD [9–11]. The action of TBX20, a member of the TBX1 subfamily of T-box proteins, is necessary in early stages of heart development,

by coordinating cardiomyocyte proliferation and regional specification and formation of cardiac chambers and valves [12]. Direct downstream target genes of TBX20 in the primitive myocardium include *TBX2* and *N-myc1* that function to regulate cardiomyocyte proliferation [13].

In adult mice, heterozygous loss of *TBX20* leads to dilated cardiomyopathy [10] and the conditional homozygous loss of *TBX20* in cardiomyocytes results in severe cardiomyopathy with associated arrhythmias and death [14]. In 2013, studies in mice showed that *TBX20* mutations resulted in failure of heart looping, developmental arrest, and lack of chamber differentiation [15].

Mutations in human *TBX20* that result in gain or loss of protein function are associated with a wide array of cardiac malformations, including septal defects, defects in valvulogenesis, and cardiomyopathy [1, 11, 16]. TBX20 carries strong transcriptional activation and repression domains, and it physically or genetically interacts with other cardiac developmental transcription factors, including NKX2-5, GATA4, GATA5, and TBX5. There have been several associations of TBX20 missense mutations with ASD. All mutations were found in the DNA-binding T-box domain, none in the transcriptional activator domain. We therefore screened 38 CHD-affected subjects for *TBX20* mutations and found three missense mutations that lay within exons encoding the transcriptional activator domain. We also found ten nonsense mutations and one nonreported SNP. These last findings could contribute to the risk of CHD.

2. Material and Methods

2.1. Study Population. Thirty-eight patients with atrial septal defect (ASD) or ventricular septal defect (VSD) attended the Department of Pediatric Cardiology at the Instituto Nacional de Cardiología "Ignacio Chávez" (INCICH). All septal heart defects were corrected with an Amplatzer septal occluder device by transcatheterization. Patients who were diagnosed with syndromatic heart defects were excluded from the study. They were unrelated individuals recruited without reference to family history during 2011-2012. Subjects included as control group underwent echocardiography to exclude CHD. Informed written consent was obtained from all recruited patients and controls. On behalf of the children enrolled in our study we obtained written informed consent from their guardians. The study complies with the Declaration of Helsinki and was approved by the Ethics Committee of the INCICH.

2.2. Mutational Analysis. Genomic DNA was isolated from peripheral blood leukocytes using standard techniques. The High Resolution Melting (HRM) method was used to detect mutations and SNPs in *TBX20* gene. The HRM primers were designed using Primer Select program (DNASTAR), which also evaluates that primers sequence do not form secondary structures during PCR that can increase the complexity of melting profile interpretation. The primers specificity was tested through PrimerBlast platform (NCBI). Primers were designed to amplify complete exonic sequences and small flanking intronic sequences (Table 1). Reactions were performed with a total volume of 20 uL (5 uL of Mili-Q water, 1.5 uL of each primer at 20 pmol/uL, 10 uL of SSoFast Eva Green Master Mix (Biorad), and 2 uL of DNA at 150 ng/uL). The amplification parameters were 95°C for 4 minutes, 30 cycles of 94°C for 30 seconds annealing temperature for 30 seconds, and 72°C for 30 seconds, followed by a final extension step of 72°C for 5 minutes. For melting curve analysis, the parameters were 95°C for 30 seconds and 75°C for 30 seconds. Data were collected over a temperature range of 75–95°C in 0.1°C increments every 10 seconds.

2.3. DNA Sequencing. After purification with Exosap, HRM products were sequenced using Big Dye Terminator v1.1 and v3.1 kits (Applied Biosystems) and ABI PRISM 3130 DNA Analyzer. Resultant sequences were analyzed with BioEdit software and nBlast platform (NCBI). Nucleotide sequences were translated using the translate tool in ExPASy Bioinformatics Resource Portal. Whenever a sequence variant was found, the sample was sequenced again from the opposite direction to confirm the nucleotide change. The effect of the mutations was evaluated *in silico*, using PolyPhen software v2.0.23 http://genetics.bwh.harvard.edu/pph2/. PolyPhen is a tool that predicts possible impact of an amino acid substitution on the structure and function of a human protein using straightforward physical and comparative considerations.

2.4. Molecular Modeling. The homology model of TBX20 was produced using the protein structure homology modeling server SWISS-MODEL and based on the structure of the T-box domain of human TBX3 (PDB 1h6f). Graphics were generated using PyMol.

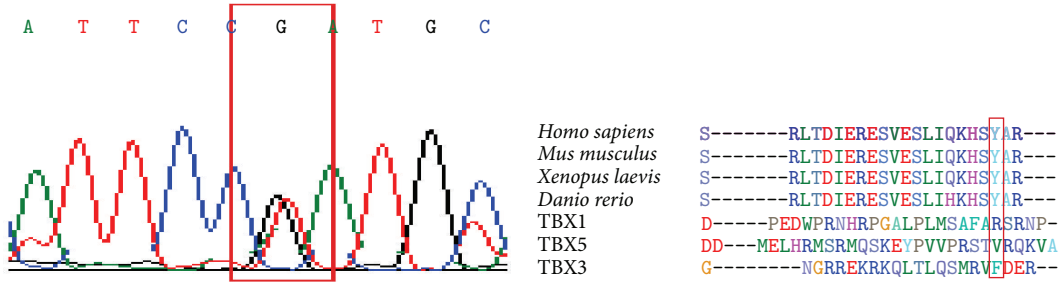
3. Results

3.1. Missense Mutations. One heterozygous transition T → G at position c.925 (NM_001077653.2) was detected in a 71-year-old female subject with ASD (Figure 1(a) and Table 2). The variant resulted in a missense mutation, a shift from tyrosine to aspartate (Y309D). It was found in 1 of the 41 cases and was not seen in the control group. This mutation was located in the transcriptional activator domain, and it is highly conserved among species, but not among other TBX proteins (Figure 1(b)). It was not possible to generate a homology model because the available templates only included 279 amino acid residues. Using program PMut, which predicts whether an amino acid substitution affects protein function, Y309D was defined as pathological mutation. With the *in silico* studies based on PolyPhen 2 (Polymorphism Phenotyping v2), we confirmed that Y309D is probably damaging.

Two heterozygous transitions were detected only in a seven-year-old female subject with ASD. The first one was a change from A → C at position c.1108 (NM_001077653.2) (Figure 2(a) and Table 2), a shift from threonine to proline in the TBX20 protein (NP_001071121.1:p.T370P) (Figure 2(b) and Table 2). The second one was a change from T → G at position c.1184 (NM_001077653.2) (Figure 3(a) and Table 2). This variant resulted in a missense change TBX20 M395R

Table 1: Exons 1 to 8 primers sequences.

Exon	Forward	Reverse
1a	5′-GATCGCCGCCGCCAGCAAAT-3′	5′-AGGAGAGGGCCCACCGAGCACTAC-3′
1b	5′-GTAGTGCTCGGTGGGCCCTCTCCT-3′	5′-GCGTTGGCCCGAGAGGAGAGTTGG-3′
1c	5′-CCAACTCTCCTCTCGGGCCAACGC-3′	5′-GCACATTCACAGCATTCAACAGAC-3′
2	5′-CATTTGGTTATGCTGTTCTTTCC-3′ [16]	5′-CTACCCAGGGAGTGTCCTG-3′ [16]
3	5′-GTTTGTGGACCGGATAGAGA-3′	5′-CAGGCTTGGAATGCTCTCTT-3′
4	5′-ACTTATATATGGTTTATGTGTT-3′	5′-GGTCCCCTGAAGAACACATAAAAT-3′
5	5′-CACTGTAATTTGGCCTGTTTAGC-3′ [16]	5′-AATATAAGAACCTCCTAAATCCTTCTC-3′ [16]
6	5′-TTCCACCCTTCTCAGGACAC-3′ [16]	5′-AGGCCTGCCTGATGTCTCT-3′ [16]
7	5′-AGTGGTTGCTTTTTGGCTGAGA-3′	5′-TCAAAGGCAAAATAATGAAATCTG-3′
8	5′-CAGTGTTTCCAGTCTAATGAGTGT-3′	5′-AGTCTGGCTCTCCTCTTTGAT-3′



(a) (b)

Figure 1: (a) The relevant sequence electropherogram of *tbx20* (NM_001077653.2) in Exon 7 of the subject. (b) The affected amino acid (Y309D) lies in a highly conserved C-terminal region of the transcriptional activator domain of TBX20. Affected region of TBX20 homologues and human TBX paralogues are shown. The variants are highlighted with a red rectangle.

(NP_001071121.1) (Figure 3(b) and Table 2). Both mutations were classified by PolyPhen software as benign (Table 2). These two mutations were located in the transcriptional activator domain and are highly conserved across species, but not among other TBX proteins (Figures 2(b) and 3(b)).

3.2. Synonymous and Noncoding Sequence Variants. Heterozygous transition C → T at position c.−517 (NM_001077653.2) was detected in a 2-year-old female subject with VSD. This variation was located in the 5'UTR region, and it was found only in this subject (Table 3).

Two synonymous variants were found in the group of patients and were absent in the control group. The first one was heterozygous transversion A → C at position c.657 (NM_001077653.2) found in three patients. The second one was heterozygous transition C → T at position c.1189 (NM_001077653.2) (Table 3) found in two patients.

Three changes that modified polyadenylation sites were found in the patients group. Three heterozygous transversions: A → T at position c.1356 (NM_001077653.2:c.*12A>T), T → A at position c.1357 (NM_001077653.2:c.*13T>A), and T → A at position c.1392 (NM_001077653.2:c.*48T>A) (Table 3).

Homozygous duplication of a timine was found in a twelve-year-old female patient with ASD, at position c.546−1223dup (NM_001077653.2) (Table 3).

Two heterozygous transversions were found in a 71-year-old female subject with ASD. The first one was at position c.1003+99C>T (NM_001077653.2) and the second one was at position c.1003+129T>C (NM_001077653.2) (Table 3).

3.3. Single Nucleotide Polymorphisms (SNPs). We also detected twelve SNPs both in CHD patients and controls. Eleven of them have been listed in database of SNP lists. Only one, the one found in intron 4, has not been reported previously (Table 4).

One heterozygous transition C → T at position c.766 (NM_001077653.2) was detected in an 11-year-old male subject with ASD (Figure 4(a) and Table 4). The variant resulted in a shift from phenylalanine to leucine in TBX20 protein (F256L) (Figure 4(b) and Table 4). This variant was already described as rs3999941 SNP. The c.766T>C was present in 9 members of his family, without any diagnosis of CHD (Figure 4(c)). As shown in Figure 5 the affected residue lies outside the DNA-binding T-box domain generating hydrophobic surface decrease of the protein (Figure 5). Analysis with PolyPhen 2 suggests that most of these missense variants are probably damaging.

4. Discussion

Most missense mutations found in TBX20 are located in the DNA-binding T-box domain [9]. The only two mutations described in the transcriptional activation domain were reported in patients with dilated cardiomyopathy [17]. We identified three unique missense *TBX20* mutations in

Table 2: Missense mutations detected in this cohort of CHD patients.

Nucleotide change	Amino acid change	Exon	Number of Patients	Cardiac defects	PolyPhen 2
c.925T>G	Y309D	7	1	ASD	D
c.1108A>C	T370P	8	1	ASD	B
c.1184T>G	M395R	8	1	ASD	B

ASD: atrial septal defect; PMut: a program predicting whether an amino acid substitution affects protein function; B: benign; D: damaging.

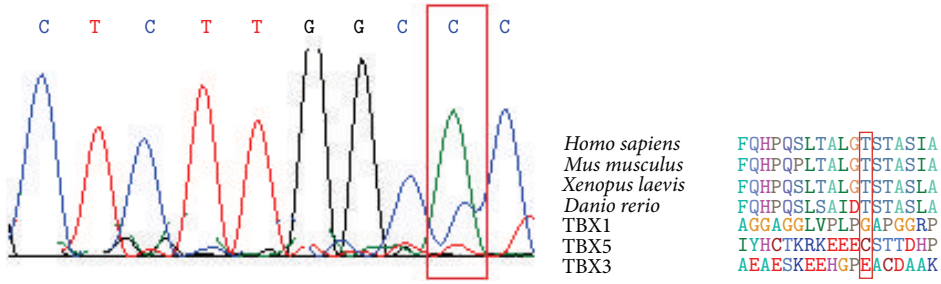


Figure 2: (a) The relevant sequence electropherogram of A → C at position c.1108 (NM_001077653.2) in the subject (Exon 8). (b) The affected amino acid (T370P) lies in a highly conserved C-terminal region of the transcriptional activator domain of TBX20 among species. The variants are highlighted with a red rectangle.

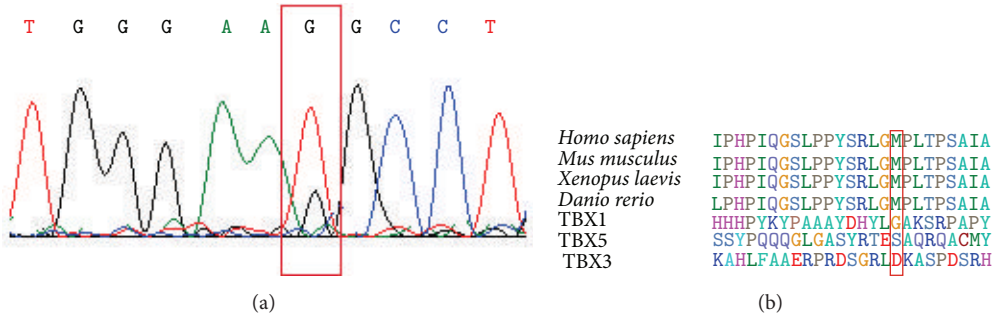


Figure 3: (a) T → G at position c.1184 (NM_001077653.2) in the subject (Exon 8). (b) The affected amino acid (M395R) lies in a highly conserved C-terminal region of the transcriptional activator domain of TBX20 among species. The variants are highlighted with a red rectangle.

Table 3: Synonymous and noncoding sequence variants in this cohort of CHD patients.

Nucleotide change	Location	Amino acid change	Number of patients	Cardiac defect
c.−517C>T	5′UTR	Non	1	VSD
c.657A>C	Exon 5	I219I	3	ASD
c.1189C>T	Exon 8	L397L	2	ASD
c.1356A>T (NM_001077653.2: c.*12A>T)	3′UTR	Non	2	ASD
c.1357T>A (NM_001077653.2: c.*13T>A)	3′UTR	Non	2	ASD
c.1392T>A (NM_001077653.2: c.*48T>A).	3′UTR	Non	1	ASD
c.546−1223dup	Intron 3	Non	1	ASD
c.1003+99C>T	Intron 7	Non	1	ASD
c.1003+129T>C	Intron 7	Non	1	ASD

VSD: ventricular septal defect; ASD: atrial septal defect; UTR: untranslated region.

Table 4: SNPs detected in this cohort of CHD patients.

Nucleotide change	Location	Effect	SNP name	Cardiac defect
c.655−18C>T	Intron 4	Non	Novel	ASD
c.−186T>C	5′UTR	Non	rs73099190	ASD
c.766T>C	Exon 5	Missense variant (F256L)	rs3999941	ASD
c.813+1G>A	Intron 6	Splicing donor variant	rs3999940	ASD
c.890+128C>T	Intron 7	Intron variant	rs2109090	ASD
c.891−55G>C	Intron 7	Intron variant	rs111666016	ASD
c.925T>A	Exon 7	Missense variant (Y309N)	rs111862418	ASD
c.891−30C>G	Intron 6	Intron variant	rs113178075	ASD
c.1164A>G	Exon 8	Synonymous variant (P388P)	rs2723759	ASD
c.1194A>C	Exon 8	Synonymous variant (T398T)	rs2532122	ASD
c.1331C>T	Exon 8	Missense variant (T444M)	rs201217462	ASD
c.655−44G>A	Intron 4	Intron variant	rs2072434	ASD

ASD: atrial septal defect; UTR: untranslated region.

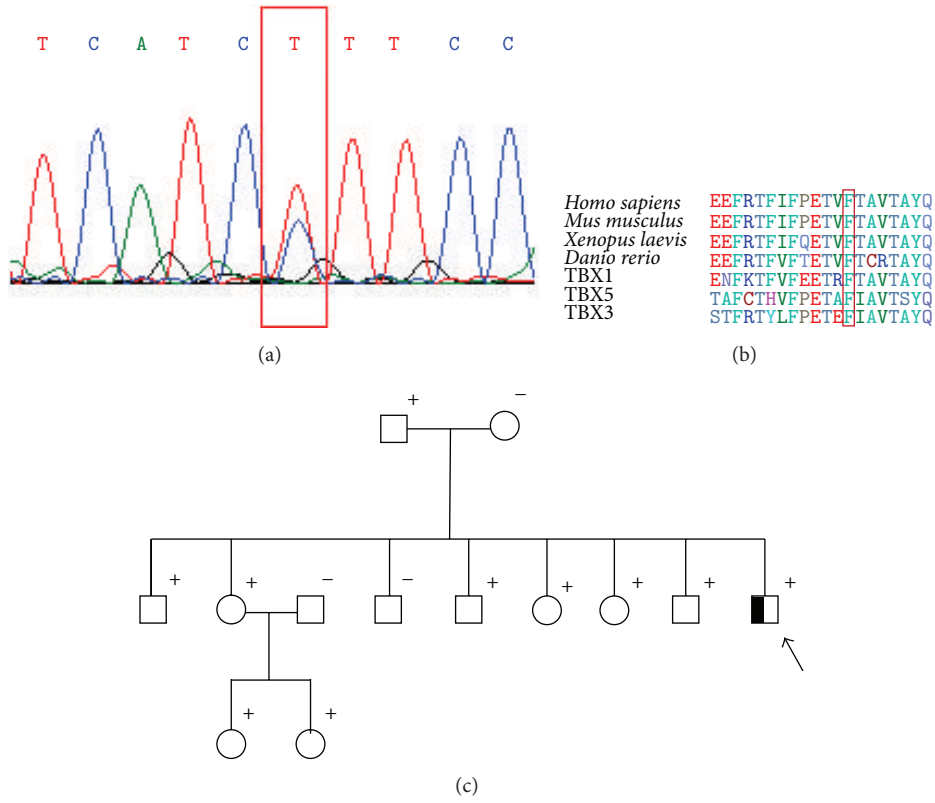


Figure 4: (a) C → T at position c.766 (NM_001077653.2) in the subject (Exon 5). The affected amino acid (F256L) is a highly conserved residue outside the DNA-binding T-box domain region of the transcriptional activator domain of TBX20 among species. The variants are highlighted with a red rectangle. (c) Family pedigree of mutation carriers. The subject is marked with an arrow. All subjects which were genotyped for TBX20-F256L are indicated with + (carrier) or − (noncarrier).

the transcriptional activation domain in two ASD subjects (Table 2). So this is the first report of *TBX20* mutations in the transcriptional activation domain in ASD patients. Neither mutation was found in our control group. These changes occurred in highly conserved amino acid among species. *In silico* analysis showed a pathological effect only of the Y309D mutation (NM_001077653.2:c.927G>T) (Table 2). This could be the result of its proximity to the DNA-binding T-box domain, so the amino acid change could have a higher effect in the protein function. The remaining two mutations (T370P and M395R) were classified by PolyPhen software as benign and are conserved across species. The three mutations could have an effect over TBX20 activity as modifiers of the affinity of the T-box domain to the T-site, because crystallographic and *in vitro* binding studies revealed that some T proteins can bind to DNA as dimers

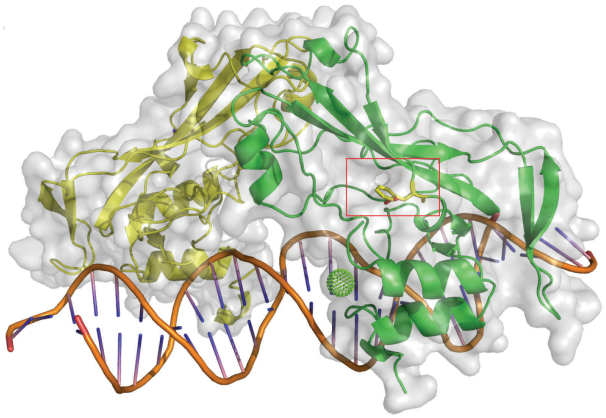


Figure 5: The homology model of TBX20, based on the structure of human TBX3, suggests that the presence of the F256L SNP diminishes in the hydrophobic surface of the protein. The variant is highlighted with a red rectangle.

and that its T-domain forms a new type of specific DNA contact, in which a carboxyterminal helix is deeply embedded into an enlarged minor groove without bending the DNA [18, 19]. Also, transcriptional activator (C-terminal region) domains of transcriptional factors are necessary to establish an interaction with the basal transcriptional machinery, and a change in this domain could affect its activity, as it was found in 2007 by Farin and colleagues. They performed a molecular analysis of TBX15 and TBX18 proteins, and they found that N-terminal and C-terminal regions participate in protein-DNA complex formation [20].

At the present time there are no outstanding findings that demonstrate the association of *TBX20* mutations with VSD. There is only one report of a missense change TBX20 I152M (456C → G) in a VSD subject [16], so in order to evaluate if there was an association in our population, we decided to include patients with VSD in the mutational analysis. Our findings showed the presence of only one noncoding change in the 5'UTR region in a subject with VSD, which was not found in the control group. As we do not know the effect of this mutation, the association between *TBX20* mutations and VSD in our population remains unclear.

The detection of synonymous, noncoding mutations, and SNPs in addition to missense mutations should be considered regarding ASD severity [21]. Some SNPs resulted in an amino acid change (c.766T>C, c.925T>A, and c.1331C>T) and one of them is a splicing donor variant (c.813+1G>A). F256L (c.766T>C) lies in a residue, which was considered as part of the DNA-binding domain [1], but the homology model of TBX20, based on the structure of human TBX3, suggests that it is located outside the T-box domain (Figure 5). The F256L seems to reduce the hydrophobic surface of the protein. Site-directed mutagenesis analysis where bulky hydrophobic residues like leucine were replaced by smaller residues as alanine demonstrated that these replacements destabilize the protein not only because there is the reduction in hydrophobic stabilization of alanine to leucine, but also because there is an energetic cost associated with the creation of a cavity in the folded protein. When a large cavity is created, the replacement is more destabilizing [22]. Despite not being part of the T-box domain, the change from phenylalanine to leucine generated by c.766T>C could affect TBX20 protein, by destabilization.

Due to its complexity it is very difficult to associate a CHD with a single mutation in a key gene in heart development, such as *TBX20*. Our findings provide the first insight into missense mutations of TBX20 transcriptional activator domain associated with ASD. Functional studies of the new variants of TBX20 identified in the present study should be subject of further investigation.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This work was supported by Research Grants from the *Consejo Nacional de Ciencia y Tecnología* (Project no. 181931). This work was submitted in partial fulfillment of the requirements for the Ph.D. degree by Irma Eloisa Monroy-Muñoz at the Graduate Studies in Biomedical Sciences Program of the *Universidad Nacional Autónoma de México*. The authors are grateful to the study participants.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 373252, 5 pages
http://dx.doi.org/10.1155/2015/373252

Research Article

A Functional Variant at miR-520a Binding Site in PIK3CA Alters Susceptibility to Colorectal Cancer in a Chinese Han Population

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Received 25 June 2014; Accepted 12 January 2015

Academic Editor: Pedro Moral

An increasing body of evidence has indicated that polymorphisms in the miRNA binding site of target gene can alter the ability of miRNAs to bind their target genes and modulate the risk of cancer. We aimed to investigate the association between a miR-520a binding site polymorphism rs141178472 in the PIK3CA 3′-UTR and the risk of colorectal cancer (CRC) in a Chinese Han population. The polymorphism rs141178472 was analyzed in a case-control study, including 386 CRC patients and 394 age- and sex-matched controls; the relationship between the polymorphism and the risk of colorectal cancer was examined. Individuals carrying the rs141178472 CC genotype or C allele had an increased risk of developing CRC (CC versus TT, OR (95% CI): 1.716 (1.084–2.716), $P = 0.022$; C versus T, OR (95% CI): 1.258 (1.021–1.551), $P = 0.033$). Furthermore, the expression of PIK3CA was detected in the peripheral blood mononucleated cell of CRC patients, suggesting that mRNA levels of PIK3CA might be associated with SNP rs141178472. These findings provide evidence that a miR-520a binding site polymorphism rs141178472 in the PIK3CA 3′-UTR may play a role in the etiology of CRC.

1. Introduction

Colorectal cancer is the third most common malignant disease worldwide and is a major cause of morbidity and mortality throughout the world [1]. During the past few decades, a rapid increase in the incidence and mortality of colorectal cancer has been reported in China [2]. Colorectal carcinogenesis is a comprehensive, multifactorial, and multistep process which is caused by the interaction of environmental agents and genetic susceptibility [3, 4]. The mechanism of colorectal carcinogenesis remains still not fully understood. Despite environmental agents found to be major risk factors for colorectal cancer, only a fraction of individuals exposed to the same risk factors develop colorectal cancer during their lifetime, suggesting that other factors were associated with the development of colorectal cancer. In recent years, an increasing body of evidence suggests that genetic polymorphisms modulate the risk of carcinogenesis and that genetic susceptibility plays an important role in the occurrence of human cancers [5, 6].

MicroRNAs (miRNAs) are a class of single-stranded 21–23-nucleotide- (nt) long endogenous noncoding RNAs that negatively regulate target gene expression at the post-transcriptional level [7]. Due to the influence on miRNA and/or their target gene expression, single nucleotide polymorphisms (SNPs) in microRNA (miRNA) genes and the miRNA binding sites at the 3′ untranslated region (UTR) of their target genes play an important role in the cancer susceptibility. PIK3CA gene that encoded the catalytic p110-alpha subunit of PI3K has been described to be commonly mutated in various cancers, including colorectal cancer [8]. A miR-520a binding site polymorphism rs141178472 was

Table 1: General characteristics of colorectal cancer cases and controls.

	Cases (n = 386)		Controls (n = 394)		P
Age (mean ± SD), years	60.1 ± 12.3		60.7 ± 12.9		0.506
Gender					
Male	216	56.0%	229	58.1%	0.563
Female	170	44.0%	165	41.9%	
Smoking					
Never	262	67.9%	276	70.1%	0.536
Ever	124	32.1%	118	29.9	
Family history of cancer					
No	332	86.0%	360	91.4%	0.023
Yes	54	14.0%	34	8.6%	
Tumor site					
Colon	212	54.9%			
Rectum	174	45.1%			
Tumor stages					
I	38	9.8%			
II	173	44.8%			
III	135	35.0%			
IV	40	10.4%			

found located at the PIK3CA 3′-UTR using bioinformatics analysis. But the association between this polymorphism and colorectal carcinogenesis remains unclear.

Given the role of microRNA and PIK3CA in carcinogenesis, we hypothesized that genetic variations in the PIK3CA 3′-UTR may confer individual susceptibility to colorectal cancer. Here, we conducted a case-control study to investigate the association of a miR-520a binding site polymorphism rs141178472 in the PIK3CA 3′-UTR with the risk of colorectal cancer in a Chinese population.

2. Materials and Methods

2.1. Study Populations. The study population consisted of 386 cases with CRC (age range, 23–72 years) and 394 controls (age range, 21–73 years). The present study is a hospital-based case-control study. Cases were the patients with pathological confirmed CRC and were consecutively recruited from Danyang People's Hospital and Zhenjiang First People's Hospital, Jiangsu. The control subjects were randomly selected from a pool of healthy individuals who got a routine health checkup. All cases and control subjects were genetically unrelated and control subjects had no individual history of cancer. All study participants signed the written informed consent. Demographic information and environmental exposure history were obtained from the participants using a standardized questionnaire. The present study was approved by the Institutional Review Board of Danyang People's Hospital and the Affiliated People's Hospital of Jiangsu University.

2.2. Genotyping. Genomic DNA was extracted with the QIAamp DNA Mini Kit (Qiagen) from isolated peripheral blood lymphocytes. The PIK3CA rs141178472 was genotyped using the allele-specific PCR assay on the S1000 thermal cycler (Bio-Rad). The primers for rs141178472 were shown in Table 1. In each 20 μL reaction, 100 ng genomic DNA was amplified by 1.5U Taq DNA polymerase (Takara) with 0.5 μL of each primer and 2 μL of 2.5 mM dNTPs. The PCR thermal cycling amplification was performed under the following conditions: 95°C for 3 min, 35 cycles of 95°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec, and 72°C for 10 min. After the amplification, electrophoresis was performed at 80 V for 60 min in 0.5X Tris-borate-EDTA buffer on 1% agarose gel stained with ethidium bromide (0.5 μg/μL). After electrophoresis, the amplified products were visualized under UV light.

2.3. PIK3CA 3′-UTR Luciferase Reporter Plasmid Construction and Transfection. The 3′-UTR region of PIK3CA containing the putative recognition site rs141178472 was amplified from a DNA sample carrying CC genotype. The primers were 5′-TCATGGTGGCTGGACAACAA-3′ (sense) and 5′-TCCAAAGCTTTACTGGTGTGAGCCACTGTG-3′ (antisense). PCR products were separated in 0.8% agarose gel, extracted, purified, and then subcloned into the pMIR-REPORT (Applied Biosystems) vector. Plasmid containing the rs141178472 T allele was generated using a QuikChange Site-Directed Mutagenesis Kit (Invitrogen). The 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, 50 U/mL penicillin, and 50 μg/mL streptomycin at a 37°C incubator supplemented with 5% CO_2. Transfections were performed with cells using Lipofectamine 2000 according to manufacturer's instruction (Invitrogen) after 24 h. The PIK3CA 3′-UTR luciferase plasmids (C allele or T allele) and chemically synthesized mature miR-520a were cotransfected into 293T cells, respectively.

Table 2: The association between rs141178472 and CRC risk.

SNP	Genotype	Cases *n* (%)	Controls *n* (%)	Odds ratio (95% CI)	*P* value
rs141178472	TT	156 (40.4)	180 (45.7)		
	CT	172 (44.6)	175 (44.4)	1.134 (0.840–1.531)	0.444
	CC	58 (15.0)	39 (9.9)	**1.716 (1.084–2.716)**	**0.022**
	T	484 (62.7)	535 (67.9)		**0.033**
	C	288 (37.3)	253 (32.1)	**1.258 (1.021–1.551)**	

After 24 h of incubation, cells were collected and processed for luciferase assay with dual-luciferase reporter assay kit (Promega Corporation).

2.4. qRT-PCR for Expression of PIK3CA. Total RNA was extracted from peripheral blood mononuclear cells (PBMCs) of 60 patients using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction (qRT-PCR) for PIK3CA was performed on Bio-Rad CFX96 Thermal Cycler using SYBR Green Q-PCR Master Mix (Takara, Dalian, China). The primers used in qRT-PCR were as follows: 5′-GGAGCCTGGAAGAGCCC-3′ (F); 5′-CGTGGAGGC-ATTGTTCTGAT-3′ (R). The qRT-PCR was performed as described previously [9].

2.5. Statistical Analysis. All statistical analyses were performed using SPSS version 12.0 (SPSS, Chicago, IL, USA) with a two-sided test. The studied polymorphism rs141178472 was tested for Hardy-Weinberg equilibrium (HWE) among the controls. The frequencies of alleles and genotypes between patients and controls were compared using χ^2 test or Fisher's exact test. Odds ratios (ORs) and 95% confidence intervals (CIs) were used to measure the strength of association between the polymorphism rs141178472 and colorectal cancer risks. $P < 0.05$ was considered sufficient for statistical significance.

3. Results

3.1. General Characteristics of the Subjects. The distributions of selected variables between cases and controls are summarized in Table 1. Briefly, there were no significant differences in the distributions of age ($P = 0.506$), sex ($P = 0.563$), and smoking status ($P = 0.536$) between the cases and controls. However, colorectal cancer cases were significantly more likely to report a family history of cancer than the controls in their first-degree relatives ($P = 0.023$). Among 213 colorectal cancer cases, 212 (54.9%) had colon cancer and 174 (45.1%) had rectal cancer. Regarding tumor stage, 38, 173, 135, and 40 patients were classified as stages I, II, III, and IV, respectively.

3.2. rs141178472 Genotype Frequencies in Cases and Control. The observed genotype frequencies of rs141178472 genetic variants sites were corresponded to Hardy-Weinberg equilibrium in controls ($\chi^2 = 0.139$, $P = 0.709$). The allelic and genotypic frequencies of the genetic variant rs141178472 were shown in Table 2. The T allele of rs141178472 genetic variant was the predominant allele in the studied subjects. As for rs141178472 C>T, significant differences were detected between the allele frequencies of CRC cases (C, 37.3%; T, 62.7%) and those of the healthy controls (C, 32.1%; T, 67.9%). In this study, we did not observe any association of relation between rs141178472 genotype and tumor stage. In addition, individuals carrying the CC genotypes for the rs141178472 were significantly associated with increased risk of CRC comparing with those carrying wild-type homozygous TT genotypes (OR (95% CI): 1.716 (1.084–2.716), $P = 0.022$). The risk of CRC was significantly higher among subjects carrying at least one C allele than among patients carrying T allele (OR (95% CI): 1.258 (1.021–1.551), $P = 0.033$).

3.3. Effect of the PIK3CA 3′-UTR Polymorphism rs141178472 on PIK3CA Expression. The rs141178472 polymorphism located at the binding site of miR-520a in the PIK3CA 3′-UTR (Figure 1(a)). As predicted using bioinformatics analysis, rs141178472 with T allele can create a new miR-520a binding site. Thus, we hypothesized that the variant T allele might lead to a reduced expression of PIK3CA resulting from increased miRNA repression. To test this hypothesis, two luciferase reporter gene plasmids containing rs141178472 T or C allele were constructed to determine whether this SNP could affect the expression of PIK3CA (Figure 1(b)). The luciferase activity of reporter gene with rs141178472 T allele was significantly lower as compared with C allele when we cotransfected chemically synthesized mature miR-520a into 293T cell ($P < 0.05$). Furthermore, we detected the PIK3CA mRNA levels in PBMCs of CRC patients. The results of qRT-PCR assay indicated that CRC patients with CC genotype had a higher PIK3CA mRNA level than that in patients with TT genotype (Figure 1(c)).

4. Discussion

In the present study, we observed an association between PIK3CA 3′-UTR polymorphism rs141178472 and risk of colorectal cancer. Furthermore, we found the polymorphism rs141178472 could affect the expression of PIK3CA. Together, our findings indicate that the miR-520a/PIK3CA axis may play a role in colorectal carcinogenesis.

An increasing body of evidence indicated that a number of cancer-associated genes can be regulated by microRNAs (miRNAs). miRNAs bind to the 3′-untranslated region (3′-UTR) of mRNA through the seed region and activate the

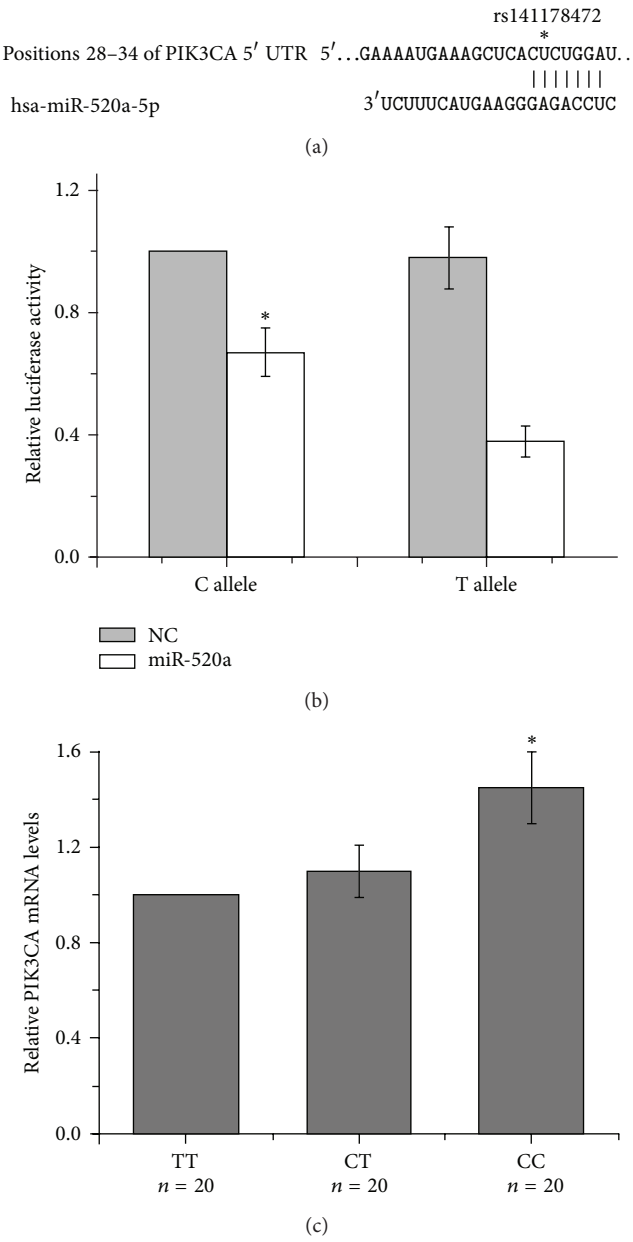

Figure 1: Effect of the PIK3CA 3′-UTR polymorphism rs141178472 on PIK3CA expression. (a) The sequence complementarity of hsa-miR-520a and PIK3CA 3′-UTR is shown here. (b) Luciferase activity of plasmids containing rs141178472 T or C allele in 293T cells. PIK3CA 3′-UTR luciferase reporter plasmids were cotransfected with chemically synthesized mature hsa-miR-520a or negative control (NC) in 293T cells. (c) The PIK3CA mRNA levels in PBMCs of CRC patients were detected by qRT-PCR assay. *Compared to TT genotype, $P < 0.05$.

degradation of mRNA, thus repressing translation [10, 11]. As the binding between mRNA and miRNA is based on complementary base pairing, a single nucleotide polymorphism (SNP) in the 3′-UTR or seed region would affect the binding efficiency, which may lead to change of the expression level of their target genes [12]. For example, the miR-184 binding site SNP (rs8126 T>C) in the 3′-UTR of TNFAIP2 could modulate TNFAIP2 expression and contributes to neck squamous cell carcinoma (HNSCC) susceptibility [13]. Hikami et al. found a SNP in the 3′-UTR of SPI1 is associated with increased SPI1 mRNA level and with susceptibility to SLE [14]. Similar results were also found in ovarian cancer [15] and breast cancer [16]. In this study, we enrolled 780 research subjects of Han ethnicity in China. We found that SNP rs141178472 in PIK3CA gene was related with the risk of CRC. These results indicated that rs141178472 might be a universal CRC-related SNP in Chinese Han population. Furthermore, the expression of PIK3CA was detected in the peripheral blood of CRC patients, suggesting that mRNA expression of PIK3CA might be associated with SNP rs141178472.

PIK3CA gene encodes for the catalytic p110-alpha subunit of phosphatidylinositol 3-kinase (PI3K) alpha, which plays a role of mediator in a wide spectrum of biological processes including cell proliferation, survival, proliferation, migration, and morphology [17, 18]. PI3K is a family of enzymes capable of 3-phosphorylating in response to activation of growth factors such as vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), or insulin [19]. PTEN is an important tumor suppressor gene and appears to negatively control the phosphoinositide 3-kinase signaling pathway [20]. The PI3K pathway is dysregulated in various cancers through either amplification or mutation in PIK3CA or inactivation of PTEN [21]. Activating mutations of PIK3CA are found most commonly in tumors of the stomach, colorectum, brain, breast, liver, ovaries, and lung [22, 23]. There are 2 mutation hotspots in the PIK3CA gene: codons 542 and 545 of exon 9 and codon 1047 in exon 20. The prevalence of PIK3CA mutations in patients with colorectal carcinoma may vary between 7% and 32%. Some studies demonstrated that PIK3CA mutations were associated with risk of cancers. He et al. found genotype CC in locus PIK3CA rs7646409 may increase the risk of osteosarcoma in the Chinese population [24]. Kommineni et al. reported 60.46% of head and neck squamous cell carcinoma (HNSCC) patients and 26% of controls with the following mutations 1634A>C (E545A) and 3075C>T (T1025T) in the helical and kinase domains of PIK3CA [25]. In the present study, we found a microRNA binding site polymorphism rs141178472 in the PIK3CA 3′-UTR is associated with the expression and risk of colorectal cancer. Our findings indicated that polymorphisms in untranslated regions of PIK3CA may play a role in colorectal carcinogenesis.

In summary, our findings indicated that miR-520a/PIK3CA axis may be involved in colorectal carcinogenesis, and rs141178472 polymorphism in PIK3CA 3′-UTR may contribute to colorectal cancer risk. Further larger studies and mechanistic investigation into the function of miR-520a/PIK3CA axis are warranted to advance the understanding of its role in colorectal carcinogenesis.

Conflict of Interests

The authors declare no conflict of interests regarding the publication of this paper.

Authors' Contribution

Lifang Ding and Zao Jiang contributed equally to this paper and should be considered co-first authors.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 769452, 7 pages
http://dx.doi.org/10.1155/2014/769452

Research Article

Gender-Dependent Effect of GSTM1 Genotype on Childhood Asthma Associated with Prenatal Tobacco Smoke Exposure

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Received 5 June 2014; Accepted 19 August 2014; Published 18 September 2014

Academic Editor: Carla M. Calò

It remains unclear whether the GSTM1 genotype interacts with tobacco smoke exposure (TSE) in asthma development. This study aimed to investigate the interactions among GSTM1 genotype, gender, and prenatal TSE with regard to childhood asthma development. In a longitudinal birth cohort in Taiwan, 756 newborns completed a 6-year follow-up, and 591 children with DNA samples available for GSTM1 genotyping were included in the study, *and the interactive influences of gender-GSTM1 genotyping-prenatal TSE on childhood asthma development were analyzed*. Among these 591 children, 138 (23.4%) had *physician-diagnosed asthma* at 6 years of age, and 347 (58.7%) were *null-GSTM1*. Prenatal TSE significantly increased the prevalence of childhood asthma in *null-GSTM1* children relative to those with *positive* GSTM1. Further analysis showed that prenatal TSE significantly increased the risk of childhood asthma in girls with *null-GSTM1*. Furthermore, among the children without prenatal TSE, girls with *null-GSTM1* had a significantly lower risk of developing childhood asthma and a lower total IgE level at 6 years of age than those with *positive* GSTM1. This study demonstrates that the GSTM1 null genotype presents a protective effect against asthma development in girls, but the risk of asthma development increases significantly under prenatal TSE.

1. Introduction

The prevalence of childhood asthma has increased worldwide in recent decades [1]. Environmental factors, including increasing air pollution, tobacco smoke exposure, a lower load of infection with pathogens, increased use of industrial materials in buildings, urbanization, and certain nutritional factors, may play an important role in this evolving epidemic. Recently, increasing evidence has demonstrated that certain types of environmental exposure may increase the risk of asthma development for certain genetic backgrounds [2], implying that the gene-environment interaction is critical in asthma development.

Oxidative stress has been implicated in the pathogenesis of asthma, which is characterized by chronic airway inflammation. The glutathione S-transferases (GSTs) are a family

of enzymes that have the general function of detoxifying xenobiotics that are capable of generating free radicals, by conjugating them with glutathione. GSTM1 has been extensively studied because its locus is polymorphic with a common null allele that produces a complete lack of the enzyme. The association between the GSTM1 null genotype and asthma development is not well established in the current literature. Several studies have demonstrated an increased risk of asthma or decreased lung function in subjects with the GSTM1 null genotype [3–9], whereas other studies have reported no association between the GSTM1 genotype and asthma [10–12]. The results of systematic reviews and meta-analyses of the effects of GSTM1 on asthma are also controversial. Some studies have revealed that the GSTM1 null genotype significantly increases the risk of asthma in children and adults [13, 14]. One meta-analysis showed that the GSTM1 null genotype may be associated with an increased risk of asthma (pooled OR 1.28; 95% CI 1.09–1.52), with large between-study heterogeneity. However, the association disappeared when the meta-analysis was repeated for the largest nine studies [15]. Another meta-analysis found no significant association between the GSTM1 polymorphism and asthma [16]. Several studies investigating the gene-environment interaction with regard to asthma development found that environmental oxidative stresses, such as tobacco smoke exposure [17–19] and ozone [20, 21], increased the risk of asthma in children with the GSTM1 null genotype but not in those with *positive* GSTM1. Another study showed that maternal use of acetaminophen in late pregnancy increased the risk of asthma or wheezing in children when the maternal or child's GSTM1 genotype was *positive* [22]. Functional studies on the role of GSTM1 in asthma are limited. Our previous studies have demonstrated that gene-gene and gene-environment interactions for IgE production begin in the prenatal stage [23–25]. This study aimed to investigate the effect of the GSTM1 genotype on the relationships among prenatal tobacco smoke exposure (TSE), childhood asthma development, and allergic sensitization for different gender backgrounds in a longitudinal birth cohort study in Southern Taiwan.

2. Methods

2.1. Study Design and Subjects. To study the effect of gene-gene and gene-environment interactions on prenatal and postnatal IgE production and the development of allergic diseases, a longitudinal birth cohort study was conducted at Kaohsiung Chang Gung Memorial Hospital, Taiwan, as reported previously [23–25]. In this cohort, the parents of 1848 children were prenatally recruited by our study nurse to enroll the birth cohort. Among the 1848 children, 1629 children were born in the hospital. In total, 1546, 1348, 1236, and 756 of the 1629 children completed the 6-month, 18-month, 3-year, and 6-year follow-up visits, respectively. DNA samples collected at the newborn stage (from the umbilical cord blood) and at 6 years of age were subjected to GSTM1 genotyping in this study. The study protocol was approved by the Institutional Review Board, and informed consent was provided to the parents at the prenatal stage. The information regarding parental atopy history and family smoking habits was obtained from a questionnaire administered during prenatal recruitment. We defined the infants as having prenatal TSE if any family member at home had a habit of smoking indoors at home. The atopy history of the children, including atopic dermatitis, allergic rhinitis, or asthma if ever diagnosed by a physician, was acquired from the questionnaire given to parents at the 6-year follow-up. Cord blood samples were collected immediately after the infant's birth for DNA collection. Blood samples were collected from children at the 6-year follow-up for DNA extraction and the measurement of allergen sensitization through the detection of specific IgE levels in response to egg whites (f1), cow's milk (f2), peanuts (f13), shrimp (f24), house dust mites (d1), and German cockroaches (i6) (Phadia CAP system) because house dust mites (approximately 90%) and German cockroaches, rather than pollen or mold (both <2%), are the major aeroallergens of children with asthma in Taiwan [26].

2.2. Analysis of GSTM1 Polymorphisms. Blood leukocytes were subjected to DNA extraction using the Gentra Puregene kit (Qiagen Inc., Valencia, CA) and then stored at −80°C after 70% alcohol precipitation.

The genetic polymorphism analysis for the GSTM1 genes was performed using an individual multiplex PCR approach [27] with the following primers: F 5′-CGCCATCTTGTG-CTACATTGCCCG-3′ and R 5′-TTCTGGATTGTAGCA-GATCA-3′ for GSTM1 and F 5′-CAACTTCATCCACGT-TCACC-3′ and R 5′-GAAGAGCCAAGGACAGGTAC-3′ for β-globin. Briefly, the multiplex PCR was performed in a 25 μL reaction mixture consisting of 50 ng of DNA, 2.5 μL of 10× GenTaq buffer, 10 mM dNTP mix, each primer at 10 μM, and 1 U of GenTaq DNA polymerase. The PCR reaction was performed in a PCR thermal cycler system (Applied Biosystems, Life Technologies) with an initial denaturation at 95°C for 5 min and then 35 cycles of 50 s at 94°C, 50 s at 60°C, and 50 s at 72°C, followed by a final elongation for 10 min at 72°C. The amplified products were visualized on a 2% agarose gel. The GSTM1 genotypes were determined by the presence or absence of bands at 230 bp, with a 260 bp internal control (β-globin). Through this simple multiplex PCR approach, the null and *positive* genotypes for GSTM1 could be clearly identified, although heterozygous and homozygous positive genotypes could not be differentiated.

2.3. Data Analysis and Statistics. The demographic data for the children with or without GSTM1 genotypes and the prevalence of allergic diseases and allergic sensitization at 6 years of age in children with or without prenatal TSE were analyzed using the chi-squared test. Multiple logistic regressive analyses were performed for childhood asthma with several factors, including preterm, gender, paternal and maternal atopy, and prenatal TSE. The log-transformed IgE levels at 6 years of age among the groups were analyzed by one-way ANOVA and Bonferroni post hoc tests.

3. Results

3.1. Demographic Data for the Birth Cohort with 6-Year Follow-Up. Of the cohort of 756 children who completed the follow-up at 6 years of age, 591 subjects, whose DNA samples collected as newborns and at 6 years of age were available, were subjected to GSTM1 genotyping in this study. Among these 591 subjects, 244 (41.3%) were *positive* and 347 (58.7%) were null for the GSTM1 genotype. In total, 188 (31.8%), 320 (54.1%), and 138 (23.4%) of these children had been diagnosed at 6 years of age with atopic dermatitis, rhinitis, or asthma, respectively, by a physician. In total, 579 of the 591 children received an allergic sensitization test at the 6-year follow-up, and 256 (43.3%) children were sensitized to house dust mites. Among the 591 children analyzed in this study, children with prenatal TSE had a significantly higher risk of developing asthma than children without prenatal TSE ($P = 0.006$, OR: 1.780, 95% CI: 1.181–2.684). However, there were no significant differences in the development of atopic dermatitis or rhinitis between children with and without prenatal TSE (Table 1). Moreover, there were no differences in food allergen sensitization or house dust mite sensitization between these 2 groups (data not shown).

3.2. Prenatal TSE Increases the Risk of Childhood Asthma in Null-GSTM1 Subjects Compared to Positive GSTM1 Children. Next, the relationship between prenatal TSE and childhood asthma in children with the GSTM1 null or *positive* genotype was analyzed. Prenatal TSE significantly increased the prevalence of childhood asthma in children with the GSTM1 null genotype ($P = 0.002$, OR: 2.337, 95% CI: 1.370–3.985) compared with those with *positive* GSTM1 ($P = 0.550$) (Table 1). Furthermore, a multivariate logistic regression analysis of childhood asthma in children with the GSTM1 null or *positive* genotype was performed to adjust for other demographic data, such as gender, prematurity, and maternal and paternal atopy. In the multivariate logistic regression analysis, male gender and prenatal TSE both significantly increased the prevalence of childhood asthma in children with the GSTM1 null genotype, whereas prenatal TSE was not a significant risk factor in children with *positive* GSTM1 (Table 2).

3.3. Gender-Dependent Association between GSTM1 Null Genotype and Asthma. To analyze the association between prenatal TSE and childhood asthma for the GSTM1 null and *positive* genotypes and in different gender backgrounds, we categorized all of the children into four groups (males with *positive* GSTM1 genotype, males with GSTM1 null genotype, females with *positive* GSTM1 genotype, and females with GSTM1 null genotype). Prenatal TSE significantly increased the risk of childhood asthma in females with the GSTM1 null genotype (30.0% versus 9.4%, $P = 0.001$, OR: 4.107, 95% CI: 1.699–10.107) compared with the effect in the other 3 groups (Table 3 and Figure 1). Furthermore, among children without prenatal TSE, females with the GSTM1 null genotype had a significantly lower risk of developing childhood asthma compared with females with *positive* GSTM1 (9.4% versus 19.3%, $P = 0.037$, OR: 0.436, 95% CI: 0.197–0.966) (Table 3 and Figure 1).

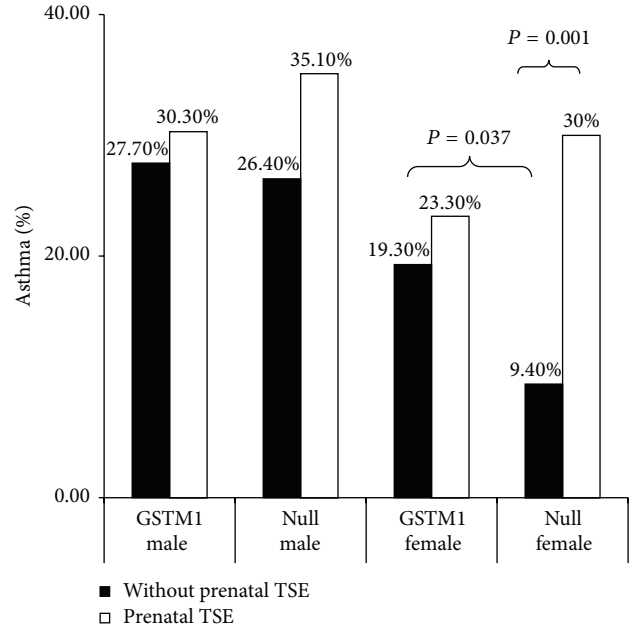

Figure 1: Interactions among GSTM1 genotype, gender, and prenatal TSE with regard to asthma development at 6 years of age. Prenatal TSE significantly increased the risk of childhood asthma in females with the GSTM1 null genotype ($P = 0.001$, OR: 4.107, 95% CI: 1.669–10.107), but not in the other 3 groups. Furthermore, among children without prenatal TSE, females with the GSTM1 null genotype had a significantly lower risk of developing childhood asthma ($P = 0.036$, OR: 0.436, 95% CI: 0.197–0.966).

3.4. Gender-Dependent Association between GSTM1 Null Genotype and Total IgE Levels. Among the children without prenatal TSE, the log-transformed total IgE level at 6 years of age was significantly lower in female children with the GSTM1 null genotype than in the other 3 groups (Figure 2). Additionally, male and female children with the GSTM1 null genotype presented lower house dust mite and food allergen sensitization compared with those with *positive* GSTM1, but the difference was not significant (Table 4).

4. Discussion

This study demonstrates that the GSTM1 null genotype could have a bipolar effect on childhood asthma development, depending on gender and prenatal TSE. In particular, the GSTM1 null genotype is a protective factor against asthma in girls without prenatal TSE but becomes a risk factor for asthma with prenatal TSE.

Asthma is a chronic inflammatory airway disorder associated with airway hyperresponsiveness and reversible airflow limitation in response to specific triggers. Disturbances in oxidation/reduction (redox) reactions and impaired antioxidant defenses have been associated with asthma [28], and lower systemic GSH levels and higher GSSG levels have been

Table 1: Allergic diseases in children with or without prenatal TSE at 6 years of age (6 y) and with different GSTM1 genotypes.

	Prenatal TSE	No prenatal TSE	*P*	OR	95% CI
6 y dermatitis	48/156	140/435	0.745	0.937	0.631–1.390
GSTM1 null	30/95	77/252	0.854	1.049	0.631–1.745
GSTM1 *positive*	18/61	63/183	0.480	0.797	0.425–1.496
6 y rhinitis	90/156	230/435	0.300	1.215	0.840–1.758
GSTM1 null	60/95	134/252	0.095	1.510	0.930–2.451
GSTM1 *positive*	30/61	96/183	0.657	0.877	0.491–1.566
6 y asthma	49/156	89/435	0.006	1.780	1.181–2.684
GSTM1 null	32/95	45/252	0.002	2.337	1.370–3.985
GSTM1 *positive*	17/61	44/183	0.550	1.221	0.634–2.348

Table 2: Multivariate regression analysis of childhood asthma in children with the GSTM1 null or *positive* genotype.

	GSTM1 null			GSTM1 *positive*		
	P	OR	95% CI	*P*	OR	95% CI
Male gender	0.003	2.317	1.342–4.000	0.126	1.606	0.875–2.950
Preterm (<37 weeks)	0.583	0.725	0.230–2.285	0.723	0.784	0.205–2.999
Maternal atopy[†]	0.488	1.235	0.681–2.240	0.595	1.206	0.604–2.409
Paternal atopy[†]	0.228	1.419	0.803–2.509	0.125	1.643	0.871–3.101
Prenatal TSE	0.003	2.308	1.333–3.997	0.553	1.223	0.629–2.377

[†]Atopy is defined by phenotypic asthma, allergic rhinitis, or atopic dermatitis along with a detectable serum specific IgE (≥0.35 kU/L) response to one or more common allergens (egg white, cow's milk, peanut, shrimp, house dust mite, or German cockroach).

Table 3: Association of prenatal TSE and childhood asthma in individuals of different genders and GSTM1 genotypes.

		Childhood asthma				
		No TSE Asthma number/total number (percentage) OR (95% CI)	Prenatal TSE Asthma number/total number (percentage) OR (95% CI)	*P*	OR	95% CI
Male	GSTM1	28/101 (27.7%) 1.000 (reference)	10/33 (30.3%) 1.000 (reference)	0.775	1.134	0.479–2.681
Male	Null	32/129 (26.4%) 0.933 (0.519–1.676)	20/57 (35.1%) 1.243 (0.495–3.121)	0.227	1.510	0.773–2.952
Female	GSTM1	17/88 (19.3%) 1.000 (reference)	7/30 (23.3%) 1.000 (reference)	0.637	1.271	0.469–3.448
Female	Null	12/127 (9.4%) 0.436 (0.197–0.966)	12/40 (30.0%) 1.408 (0.477–4.159)	0.001	4.107	1.669–10.107

Table 4: The GSTM1 null genotype presented a lower prevalence of house dust mite sensitization and food allergen sensitization compared with *positive* GSTM1 in boys and girls without prenatal TSE, but the difference was not significant.

	Male					Female				
	GSTM1		Null		*P*	GSTM1		Null		*P*
6 y HDM sensitization[*]	51/100	51.0%	57/126	45.2%	0.389	37/87	42.5%	48/122	39.3%	0.644
6 y food allergen sensitization[#]	27/100	27.0%	31/126	24.6%	0.682	22/87	25.3%	23/122	18.9%	0.265
6 y any sensitization[†]	59/100	59.0%	66/126	52.4%	0.32	42/87	48.3%	52/122	42.6%	0.418

[*]HDM sensitization is defined by a specific IgE response to house dust mites (d1) ≧0.35 kU/L.
[#]Food allergen sensitization is defined by a detectable serum specific IgE (≥0.35 kU/L) response to one or more food allergens (egg white, cow's milk, peanut, or shrimp).
[†]Any sensitization is defined by a detectable serum specific IgE (≥0.35 kU/L) response to one or more common allergens (egg white, cow's milk, peanut, shrimp, house dust mite, or German cockroach).

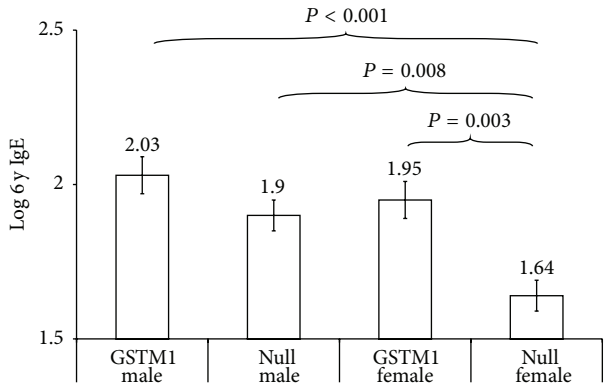

Figure 2: Interaction of the GSTM1 genotype and gender with regard to the log-transformed total IgE level at 6 years of age among individuals without prenatal TSE. Among children without prenatal TSE, the log-transformed total IgE level at 6 years of age was significantly lower in females with the GSTM1 null genotype than in the other 3 groups. The results are presented as the mean ± standard error.

reported to be related to asthma development and increased asthma severity [29–31]. GSTM1, one of the most studied enzymes in the GST family, functions in the detoxification of xenobiotics, environmental toxins, and products of oxidative stress by conjugation with glutathione, thereby protecting cells from reactive oxygen species (ROS). Individuals with the GSTM1 null genotype lose GSTM1 enzymatic activity and thus may be vulnerable to oxidative stress in the airway and have a higher risk for airway inflammation. Most previous studies have reported that the GSTM1 null genotype significantly increases the risk for asthma development, although some studies found this risk to be insignificant. Wu et al. found that stimulation of primary human bronchial epithelial cells (*positive* GSTM1) with diesel exhaust particles (DEP) significantly increased IL-8 and IL-1β protein expression, and knockdown of GSTM1 in these cells further elevated DEP-induced IL-8 and IL-1β expression, implying that GSTM1 deficiency aggravates DEP-induced proinflammatory responses [32].

Similar to a previous study showing that the GSTM1 null genotype increases the risk of childhood asthma only in children with a history of intrauterine smoke exposure [33], this study found that the GSTM1 null genotype is associated with a higher incidence of asthma than the *positive* GSTM1 genotype is in boys and girls with prenatal TSE, with a nonsignificant difference (35.3% versus 30.3%, $P = 0.643$, OR: 1.243, 95% CI: 0.495–3.121 for boys, 30.0% versus 23.3%, $P = 0.535$, OR: 1.408, 95% CI: 0.477–4.159 for girls). Additionally, we found that prenatal TSE increases the risk of childhood asthma in individuals with the GSTM1 null genotype, which supports the findings of other studies [17–19]. Furthermore, we found that prenatal TSE significantly increases the risk of childhood asthma in girls with the GSTM1 null genotype, but not in boys with the GSTM1 null genotype.

Among the children without prenatal TSE, girls with the GSTM1 null genotype had a significantly lower incidence of asthma and lower log-transformed IgE level at 6 years of age than girls with *positive* GSTM1. Additionally, among individuals (either boys or girls) without prenatal TSE, the GSTM1 null genotype was associated with lower house dust mite or food allergen sensitization, but the difference was not significant. These results suggest that the GSTM1 null genotype may protect against the development of asthma in girls if no prenatal TSE is present. This hypothesis is supported by a study in which the GSTM1 null genotype is associated with a decreased risk for asthma among atopic subjects [34] and another study investigating the effect of the *positive* and null-GSTM1 genotypes on allergen-induced oxidant stress, airway inflammation, and reactivity in vivo in adults with mild atopic asthma and without a regular asthma medication [35]. In this study, patients with a *positive* genotype had higher baseline and allergen-provoked airway neutrophilia and higher concentrations of myeloperoxidase than GSTM1 null patients. The allergen-stimulated generation of the acute-stress and proneutrophilic mediators tumor necrosis factor-α, CXCL-8, IL-1β, and IL-6 and the postallergen airway concentrations of IgE and the neutrophil-generated mediators matrix metalloproteinase-9, B-cell activating factor, transforming growth factor-β1, and elastase were also higher in patients with *positive* GSTM1. This study also found that GSTM1 *positive* individuals with asthma were more reactive to specific allergens, producing a 20% decrease in FEV1, but eosinophil inflammation and allergen-induced F2-isoprostane levels, which are considered to be specific markers of oxidative stress in vivo, were unaffected. These findings imply that certain phenotypes of asthma may be affected by the GSTM1 genotype.

There are certain limitations in this study. Not all children who completed the 6-year follow-up had DNA samples qualified for GSTM1 genotyping, making selection bias possible. However, the demographic data on the 591 and 165 children with and without available DNA samples, respectively, including data on parental allergic diseases and sensitization, gender, prematurity, and the presence or absence of prenatal TSE, were not significantly different (data not shown). Moreover, our study examined TSE at the prenatal stage but not in the infant stage or childhood stage. *Furthermore, we acknowledge that caution must be used in generalizing these results to other populations, because the GST loci strongly interact with the environment. The different environmental risk factors, which are present in human populations, may certainly influence the results of this type of genetic association studies.*

This study yields additional evidence for the gene-environment interaction with regard to the development of childhood asthma and IgE level, starting in the prenatal stage. Based on the present findings, the GSTM1 null genotype in girls may be a protective factor against childhood asthma development and IgE level. However, girls with the GSTM1 null genotype are much more vulnerable to environmental oxidative stresses, such as prenatal TSE. The mechanism may not involve simple redox equilibrium because the GSTM1 genotype (null or *positive*) was previously found to be unrelated to the total plasma GST enzymatic activity, and

the lack of GSTM1 activity for the GSTM1 null genotype may be compensated by other members of the GST superfamily [36]. Similarly, another study also found that *positive* GSTM1 was possibly related to neutrophilic inflammation in asthma without affecting allergen-induced F2-isoprostane levels [35]. Further studies are necessary to investigate the exact mechanism by which the GSTM1 genotype influences airway inflammation and allergic responses.

5. Practical Implications

This study demonstrates a gene-environment-gender interaction in the development of childhood asthma. Specifically, prenatal TSE increases the prevalence of childhood asthma. The GSTM1 null genotype is a protective factor against asthma development in girls without prenatal TSE but becomes a risk factor with prenatal TSE.

Disclosure

Chih-Chiang Wu and Chia-Yu Ou are joint first authors.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

This study was supported in part by funding from Grants NSC 97-2314-B-182A-054-MY2 and NSC98-3112-B-182-004 (97IR007) from the National Science Council of Taiwan; Grants CMRPG860422, CMRPG850353, and CZRPG880253 from the Chang Gung Memorial Hospital, Taiwan; and Grant CMRPRA11024 from the Show Chwan Memorial Hospital, Taiwan. The authors thank the staff of the delivery room and outpatient clinics of Kaohsiung Chang Gung Memorial Hospital, Kaohsiung, Taiwan, for the collection of blood samples in this cohort study. The authors acknowledge the technical support provided by the Genomic & Proteomic Core Laboratory, Department of Medical Research, Kaohsiung Chang Gung Memorial Hospital.

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