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Original Article

Single nucleotide polymorphisms of ERβ and coronary atherosclerotic disease in Chinese Han women

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Abstract: Background: Growing evidence has shown that with the increase of age, the incidence of coronary atherosclerotic disease (CAD) in women increases to equal that of men. Several studies on the single nucleotide polymorphisms [SNPs] seem to provide evidence in support of the protective role estrogen receptor β (ERβ) has in reducing the risk of CAD. Objective: To determine the association of ERβ SNPs rs1256049 RsaI 1082 A > G and rs4986938 AluI 1730 G > A with coronary atherosclerotic disease in Chinese Han women. Methods: We designed a nested case-control research, in which 120 case women and 30 control women were selected from the Forensic Medicine Department of Tongji Medical College, HUST. We isolated DNA from their lung paraffin blocks, and then screened for these two SNPs for each DNA sample. Post-statistical analysis of their genotypes and haplotypes was used to figure out the targeted association. Results: We found no significant difference between the genotypes or haplotypes of the two SNPs and the risk of CAD. However, the rs4986938 heterozygote AG variant was correlated with a significantly lower risk for CAD than did homozygote GG variant in the group of less than 40 years old. Haplotype AA of the two SNPs was correlated with a higher risk for CAD in the same group. Conclusion: The rs4986938 AluI 1730 G > A seems to be quite involved in the genetic basis of the disease and needs more attention in future studies. Meanwhile, this very association made between CAD and the mentioned SNP seems to be affected quite a bit by age.

Keywords: ERβ, SNP, CAD, rs4986938, Chinese Han women

Introduction

It is well known that coronary atherosclerotic disease (CAD) has significant gender differences: the incidence in men is generally higher than in pre-menopausal women, and equal to the incidence in postmenopausal women. Epidemiological statistics from a recent study have showed that cardiovascular mortality in men is three times more than in women (3:1) [1]. And estrogen has been found to be responsible for this distinction. In regards with this, two isoforms of the estrogen receptor (ER) α and β have been identified. The exact effect they have on the cardiovascular system is still unclear. The aim of this study was to reveal whether there is an association between CAD and ERβ SNPs rs1256049 RsaI 1082 A > G and rs4986938 AluI 1730 G > A by performing a nested case-control research on 150 Chinese Han women residing in central China area. rs1256049 and rs4986938 were selected as target SNPs for ERβ in this investigation.

Materials and methods

Cases and samples

In this nested case-control research, 150 cases were randomly selected from the files of Department of Forensic Medicine, Tongji Medical College, Huazhong University of Science and Technology (HUST), the cases, chosen from the records between 2009 to 2013, were divided into two groups, experimental and control. The experimental group consisted of 120 cases of coronary atherosclerotic heart disease, CAD in individuals aged between 21 and 84 (52.4 ± 14.9). The control group consisted of 30 non-CAD affected cases aged between 20 and 62

Table 1. Genotype distribution and allele frequencies of ERβ polymorphisms among CAD cases and controls

SNP	Control	CAD	P
rs1256049 [n]	23	87	
GG	8 (34.8)	34 (39.1)	
GA	9 (39.1)	33 (37.9)	
AA	6 (26.1)	20 (23.0)	0.955
G	13 (56.5)	51 (58.6)	
A	10 (43.5)	36 (41.4)	0.955
rs4986938 [n]	28	89	
AA	3 (10.7)	13 (14.6)	
AG	10 (35.7)	31 (34.8)	
GG	15 (53.6)	45 (50.6)	0.912
A	8 (28.6)	29 (32.6)	
G	20 (71.4)	60 (67.4)	0.869

Data are n (%).

(38.8 ± 11.5). All cases were autopsied within the last 5 year and we further divided them into different subgroups according to their age. The criteria for acceptance into the experimental group was for the deceased to have a severe atherosclerotic ≥ 50% stenosis in the cross section of at least one branch of coronary artery [2], the standard used in several publications and clinical practice [3], and to not have been part of violent causes of death such as mechanical injury and poisoning. The 30 cases in control group had no signs of severe coronary atherosclerotic disease and had died from mechanical injury or poisoning. All samples came from the paraffin masses of these deceased's lung. The reason for isolating DNA from lung paraffin masses was due to the tissue's ability to resist degradation for longer than other tissues, and it being relatively easier to screen for SNPs in lung tissue.

Methods

DNA was isolated from paraffin tissue using a kit [FFPE Tissue DNA Purification Kit, DTK-01-250]. The target fragments containing the required SNPs were amplified from the genomic DNA extracted from embedded tissues. The PCR products were analyzed directly by a sequencing system [dideoxynucleotide-sequencing method ABI BigDye 3.1v; Sequencing analyzing v5.2]. The SNPs of interest were identified in the ERβ gene: rs1256049, or RsaI, SNP 1082 A→G transition in the ligand binding region located in exon 5, using the forward and reverse primers 5'-GGATTGGGTCAGACAGGCAG-3' and 3'-AATTGCAGCACCCAGGACTT-5'. rs4986938, or AluI, a 1730 G→A transition in the non-translated region downstream of exon 8, using the forward and reverse primers 5'-GTTGCGCAGCTTAACTTCAAA-3' and 5'-TGTTCCCACTCACTAAGCACC-3'.

Statistical analysis

The χ^2-analysis and Fisher exact test were used for comparison between genotype and allele frequencies while deviation was assessed by using Hardy-Weinberg equilibrium. Pairwise linkage disequilibrium was preliminarily examined by χ^2-analysis. Haplotype frequencies were estimated from genotype data using the PHASEv2.1.1 algorithm [4]. Haplotype distributions between cases and controls were compared by global likelihood ratio test. For each odds ratio, 95% of CIs was calculated. A 2-tailed nominal *P*-value of 0.05 was considered to represent a statistically significant result.

Genotype distribution and allele frequencies between cases and controls were compared by Fisher exact test, P > 0.05 shows no significant differences exist among them (**Table 1**). By calculating odds ratios and the corresponding 95% confidence intervals between two different genotypes among cases and controls, we can figure out which genotype has significant higher or lower risk for SAD in different age levels (**Table 2**). The Chi-square test was used to test the difference between estimated and expected haplotypes of rs1256049 and rs4986938, which presents if the distribution of these haplotypes complies with the law of inheritance (**Table 3**). Odds ratios and the corresponding 95% confidence intervals between individual haplotype and others in different age groups can reveal which haplotype has higher or lower genetic risk than others (**Table 4**).

Results

Screening the SNPs revealed the rs1256049 to be present in 87 CAD cases and 23 controls and rs4986938 to be present in 89 CAD cases and 28 controls (**Table 1**). Genotype frequencies of rs1256049 didn't differ significantly from the predicted frequencies obtained using Hardy-Weinberg equilibrium [P = 0.070]. How-

Table 2. ORs and 95% CI for the effect of ERβ polymorphisms on the risk of CAD in different ranges of age

	SNP	OR	95% CI	*P*
rs1256049				
Overall	AA/G	0.91	0.28-2.94	0.874
	AA/GG	0.78	0.24-2.59	0.690
	A/GG	0.86	0.30-2.51	0.786
~40	AA/G	2	0.37-10.92	0.800
	AA/GG	1.43	0.24-8.64	0.698
	A/GG	0.71	0.16-3.23	0.662
40~60	AA/GG	0.33	0.05-2.12	0.244
rs4986938				
Overall	AA/G	0.72	0.17-3.03	0.649
	AA/GG	0.69	0.17-2.76	0.603
	A/GG	0.97	0.39-2.43	0.944
~40	AA/G	2.89	0.32-25.70	0.341
	AA/GG	0.39	0.05-2.92	0.359
	A/GG	0.13	0.03-0.64	0.012 < 0.05
40~60	AA/G	0.21	0.02-2.09	0.185
	AA/GG	1.19	0.09-15.03	0.855
	A/GG	5.54	0.98-31.25	0.052

ever, the genotype frequencies proved to be significantly different from the predicted in the case of rs4986938 [P = 0.002]. In addition, no significant differences were found in genotype frequencies for both rs1256049 and rs4986938 between CAD and control group, and the same applied for the allele frequencies of these subjects (**Table 1**). These data indicate that there is no correlation between different genotypes and the incidence of CAD for both rs1256049 SNP and rs4986938 SNP. The nonexistence of any association between SNP genotypes and CAD subjects was affirmed when no relative risks were detected among the different genotypes for these two SNPs (**Table 2**).

Further analysis on three different age groups suggested that there is a lower odds-ratio [0.13, 95% CI 0.03-0.64] for patients carrying AG at rs4986938 than those carrying GG at age ≤ 40. In addition, no significant relative risks were found between different genotypes in other age groups for the both SNPs (**Table 2**). For rs4986938, we found that the most significant difference exists between the heterozygote carriers and the GG carriers at the age of 40 and less. No such relations were found in other age groups and in SNP of rs1256049.

Chi-square test for the frequencies of 4 haplotypes estimated the recombination of rs1256049 and rs4986938, hence, no linkage disequilibrium was found between the 2 SNPs (**Table 3**). After haplotype analysis in overall subjects and 3 different age groups, however, it was discovered that haplotype AA carriers under age 40 had, to some extent, an association with CAD at a relative risk of 6 [95% CI 1.23-29.14]. Concurrently, no such correlation was detected among other haplotypes in these age groups (**Table 4**).

Discussion

We found, during the course of this study, no statistically significant difference of genotype and allele frequencies between CAD cases and control. The results indicated that no particular genotype had predisposed the subjects to a higher risk of CAD. Moreover, further statistical analysis of the data revealed that none of the haplotypes in this study had any significant difference in the incidence of CAD.

In 2009, Xing and his colleagues reported that the estrogen exerts its cardiovascular protective effects through receptor-mediated biological mechanisms [5]. Since the classical structure of ERα was identified in 1987, many investigators around the world have been working to further understand its function and coding polymorphisms. However, the studies related to ERβ are much fewer, in part because it was only recently cloned in 1996.

As isoforms of estrogen receptors, α and β are widely distributed in the reproductive system, heart, vascular endothelial cells and vascular smooth muscle cells [6]. They seem to have different levels of expression in different organs or tissues. ERα and β both belong to the nuclear receptor super family and are highly homologous. The N-terminal of ERβ has a DNA binding domain to identify a specific gene sequence, which is a secondary structure consisting of two zinc fingers. Each of these fingers contains 4 α helixes composed of cysteine and zinc ions. On the other hand, C terminal has an identification binding region for another specific lig and that is a tertiary structure formed by 12 α-helixes and 2 8-layer foldings [7].

Chambliss et al. found that almost all the biological reaction that leads to the activation of

Table 3. Estimated and expected population of ERβ haplotypes

Haplotype	rs1256049	rs4986938	estimated	expected
1	G	A	33	41.56
2	G	G	94	85.44
3	A	A	39	30.44
4	A	G	54	62.56

P = 0.102.

the endothelial NO synthase [eNOS] generating NO and subsequently leading to vasodilation was due to ERβ [8]. In addition, Karas et al. reported a significant increase in the expression of ERβ mRNA located in smooth muscle and endothelial cell after vascular injury, with noticing no alteration in the expression of ERα [9]. This pointed towards the dominant role that ERβ mediated direct effects of estrogen had on blood vessels during vascular injury [9]. Furthermore, it has been shown that ERβ is extensively expressed in the human coronary system, and in the event of severe atherosclerosis and calcification, faces a significant increase in expression [10]. Contrary to what has been published earlier, Ortmann et al in 2011 reported that it is ERα, not ERβ that plays a major role in vascular protection [11].

In 2007, Babiker et al. reported that myocardial infarct size in ERβ knockout mice with estrogen replacement therapy was significantly greater than WT [12]. In a following study, Paula et al concluded that ERβ should be the isoform that mediate protective effects of estrogen on myocardial infarction [13]. Further research found that the mortality rate of post-myocardial infarction was likely to be reduced by ERβ selective agonists through improvement of left ventricular dysfunction. Recently, the balance between these two receptors has been found to play a key role in regulating response of myocardial cells to estrogen receptors [6]. In addition, ERβ knockout mice are characterized by ventricular hypertrophy [14] and systemic hypertension [15]. Studies has also shown that ERβ can decrease the hypertrophic response to pressure overload [16] and protect the heart against ischemia/reperfusion injury [17, 18].

There have been several studies on the relationship between polymorphisms and haplotypes for ERβ and the risk of CAD. In 2005, Mansur AP, by studying polymorphisms of four human estrogen receptor genotypes in a group of early coronary heart disease patients, found that ERβ point mutation was an independent risk factor for coronary heart disease [19]. However, the existence of different ERβ gene polymorphisms between different ethnic groups makes ERβ's relation with coronary heart disease very confusing. Domingues concluded that rs1271572 SNP T variant for ERβ was associated with increased risk of MI in the Spanish population, and while this association was found to be limited to men only, the rsl256049 and rs4986938 variants seemed to have no association with the risk of MI in this same population [20]. In contrast, Rexrode et al. found that rsl256049 protected North American white females against CAD [21]. Furthermore, in a Brazilian case control study, an ESR2 variant, rs4986938 was found to be more common among cases with premature coronary artery disease than among controls [19]. So far, there had been no study reported aiming to identify the association between polymorphisms of ERβ and the risk of CAD in China.

In this study, we investigated the correlation between two single nucleotide polymorphisms of ERβ, rs1256049 and rs4986938, and the risk of CAD in Han women living in central China. For both of these SNPs, we found no significant difference of genotype and allele frequencies between CAD cases and control. The results indicated that no particular genotype had preordained the subjects to a higher risk of CAD. Moreover, further statistical analysis of the data on haplotypes revealed that none of these haplotypes had any significant difference in the incidence of CAD. To some extent, these results showed that no clear association existed between the two SNPs of ERβ and CAD, which is similar to what was found in Domingues's study [20]. In his research, by screening 3 SNPs of ERβ in a nested case-control design in a population of Spanish people, he found no association between MI and rs1256049 or rs4986938 [20]. On the other hand, in another study related to North American women, they found that women with the relatively rare A allele of rs1256049 had decreased susceptibility to CAD and MI [21]. These differences seem due to different ethnicities. Until now, however, no study has been reported about the association between the SNPs of ERβ and CAD among the Han population. In

Table 4. Association of ERβ haplotypes and CAD in different ranges of age

	Haplotype	OR	95% CI	*P*
Overall	GG	1.64	0.83-3.26	0.157
	GA	0.57	0.25-1.31	0.189
	AA	1.92	0.70-5.23	0.202
	AG	0.56	0.28-1.13	0.105
~40	GG	1.30	0.50-3.37	0.592
	GA	0.48	0.14-1.69	0.255
	AA	6	1.23-29.14	0.026 < 0.05
	AG	0.39	0.14-1.12	0.081
40~60	GG	2.32	0.58-9.23	0.233
	GA	0.84	0.20-3.43	0.804
	AA	0.60	0.14-2.52	0.486
	AG	0.66	0.16-2.74	0.563
60~	GG	0.93	0.06-15.62	0.959
	AG	0.50	0.03-8.46	0.631

2010, Teng Zhao and his colleagues examined the association between ESR1 and ESR2 gene polymorphisms and hyperlipidemia in Chinese Han postmenopausal women [22]. As an important risk factor of CAD, hyperlipidemia was studied in relation to the SNPs of ERβ. In that study, a total of 443 postmenopausal women aged between 55 and 71 years were recruited from Shanghai, China for a case-control study [154 women with hyperlipidemia and 289 controls]. They found that rs1256049 and rs4986938 showed no statistical association with hyperlipidemia.

Since we did not find an association of the two SNPs with CAD, we further subdivided the genotypes and haplotypes into 3 different age groups. Within each age group, most of these genotypes and haplotypes were found to have no significant association with the risk for CAD. However, as an exception, we found that the rs4986938 heterozygotes AG carrier had a significantly lower risk for CAD than GG carrier in the group of less than 40 years old. Meanwhile, the haplotype of AA was found to have a much higher risk of CAD than others in the same age group. Accordingly it was concluded that the allele A for rs4986938 may have a significant influence on the incidence of CAD among women patients who were younger than 40-years old. This area needs to be further explored and the corresponding allele's role should be studied among the Han population.

The risks for CAD are not only gender dependent, but are also affected by age. As mentioned earlier, the incidence of CAD in older men is similar to that in postmenopausal women [23, 24]. There have been many studies using menopausal hormone therapy to investigate the role of estrogen and its receptors in CAD. An epidemiological study on the Far East population has revealed that dietary intake of phytoestrogens may contribute to the decreased incidence of postmenopausal CAD and thromboembolic events [25]. In addition, a study by Women's Health Initiative [WHI] restricted to younger postmenopausal women showed that initiation of hormone replacement treatment [HRT] close to the women's menopause reduced the risk of CAD [26]. These studies show that when studying the association between the polymorphisms of ERβ and the risk of CAD one should not ignore the influence of age on this relationship. Besides, as other researchers inferred, the translation to RNA might be affected if the SNP belongs to a site not yet described, such as transcription factor binding sequences. Meanwhile, the observed association of the SNP with MI may also be related to a change in the structure of either the DNA or RNA molecules, e.g., a change in the ability of the DNA to wrap itself into a nucleosome around histones, thus affecting the access of regulatory proteins to their binding sites [27]. Although we found an association with CAD of the rs4986938 heterozygote AG and one of its haplotypes AA in the less than 40-year old group, we still cannot draw an absolute conclusion regarding the role these two SNPs have on the risk of CAD among Han women. Nevertheless, Herbert and his colleagues suggested that genotyping for the rs4986938 G > A gene variant should be included in a screening panel for assessment of cardiovascular risk in menopausal women according to the data from their studies in Sicilian menopausal women [28].

ERβ is more active in the prostate and ovaries, and less active in the lungs, brain, and bones [24]. Particularly in women, it is the estrogen receptor that is predominantly expressed in human vascular smooth muscle [29]. As a leading risk factor for CAD, hypertension has also been taken into consideration when studying the role of ERβ. On the contrary to the controversy raised regarding GPER-1 and estrogen signaling in the membrane by Levin and coll-

eagues, some studies concluded that the main protector against CAD was ERα or GPER-1 instead of ERβ [30, 31]. At the same time, several other investigators supported the theory that the key isoform that has a protective effect against the risk of CAD is the ERβ [32].

In conclusion, we found that rs4986938 G > A has an association with the risk of CAD in Han women under 40 years old. Its exact role remains to be determined. These findings are important to study the genetic basis and the complex mechanisms that underlie coronary atherosclerotic disease. Further studies are needed that can combine functional explorations, polymorphisms and molecular regulation pathway to explain the exact contribution of this variant in cardiovascular disease among the Han population.

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Disclosure of conflict of interest

None.

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