Clinical Importance of *Estrogen Receptor 1 (ESR1)* Gene Polymorphisms and Their Expression Patterns in Coronary Artery Disease Patients: A Study from India

Mamta P. Sumi¹ · Sameer Ahmad Guru¹ · Rashid Mir¹ · Mirza Masroor¹ · Musadiq A. Bhat¹ · M. P. Girish² · Alpana Saxena¹

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Abstract The influence of Estrogen Receptor 1 (ESR1) gene -397T>C (PvuII) and -351A>G (XbaI) polymorphisms on the risk of development of coronary artery disease (CAD) in the north Indian population was analysed. We hypothesized that ESR1 gene polymorphisms may influence the susceptibility to CAD through variation in Estrogen Receptor α (ER α) expression. To assess this concept, we evaluated ER α mRNA expression in blood plasma of CAD patients. The study included hundred CAD patients who showed presence of greater than 50% luminal stenosis in at least one major coronary artery in angiography along with hundred age and sex matched healthy controls. The ESR1 polymorphisms were investigated by PCR–RFLP. Quantitative Real Time PCR was carried out for the measurement of ER α mRNA expression. The results showed that genotypic frequencies of ESR1 -397T>C and -351A>G gene polymorphisms were significantly higher in CAD patients than control subjects ($p < 0.0001$). A significantly increased CAD risk was also found in dominant and codominant inheritance model for both of the SNPs. In gender based analysis these findings were replicated only in male subgroup. In case of -397T>C polymorphism, the ER α mRNA expression was highest in CAD patients with wild type homozygous TT genotype ($2^{-\Delta\text{ct}} = 0.28$). A mutant ‘C’ allele, dose dependent, significant decrease in trend in ER α mRNA expression was observed, with lowest expression in mutant homozygous

CC genotype ($2^{-\Delta\text{ct}} = 0.09$), and intermediate expression level in heterozygous TC genotype ($2^{-\Delta\text{ct}} = 0.14$) subgroups of CAD patients. In conclusion, this study demonstrates a significantly heightened risk of CAD associated with the inheritance of mutant genotypes of ESR1 -397T>C and -351A>G gene polymorphisms, in the north Indian population. This is the first report of a lowered ER α mRNA expression in conjunction with the presence of mutant ‘C’ allele of ESR1 -397T>C polymorphism with consequent increased CAD susceptibility.

Keywords Coronary artery disease · ESR1 gene polymorphisms · ER α mRNA expression · Real time PCR

Introduction

Coronary artery disease (CAD), the most common chronic disease in humans, has an extremely complex etiology. A number of studies have demonstrated that the incidence of CAD and its sequelae such as myocardial infarction (MI) is lower in pre-menopausal women than in age-matched men [1]. However, cardiovascular morbidity and mortality may increase rapidly after the onset of menopause. This suggests that endogenous estrogens may have a protective role in women against CAD [1]. Further, there are reports which indicate that women who used postmenopausal hormone replacement therapy exhibited a lower cardiovascular disease related morbidity/mortality than non-users [2]. Considering these factors, it has been suggested that estrogens, mostly 17 β -estradiol (E2), may play an important role in cardioprotection. E2 performs its biologic functions by interaction with its receptor proteins. The first known E2 receptor was isolated by Toft and Gorski from the rat uterus in 1966 [3]. The human Estrogen receptor

✉ Alpana Saxena
alpanasaxena346@gmail.com

¹ Department of Biochemistry, Maulana Azad Medical College, University of Delhi, New Delhi, India

² Department of Cardiology, GB Pant Hospital, University of Delhi, New Delhi, India

(ER) cDNA was first cloned in 1985; it encodes the ER isoform presently known as ER α [4]. The second human isoform of estrogen receptor ER β , was cloned in 1996 [5]. ER α and ER β in humans are encoded by different genes, *Estrogen Receptor 1 (ESR1)* and *Estrogen Receptor 2 (ESR2)* respectively, although they share high structural homology. *ESR1* is located on chromosome 6 and *ESR2* is located on chromosome 14 in human [6].

ER α and ER β differ in their biological functions though both bind to their ligands with high affinity in a similar fashion. Estrogen signalling pathways are selectively stimulated or inhibited depending on a balance between activities of estrogen receptor ER α or ER β in target organs. There are major differences between ER α and ER β with respect to their tissue distribution and transcriptional activities [6]. There is accumulating evidence that both ER α and ER β estrogen receptors have a crucial role in maintaining homeostasis of cardiomyocytes and in cardio protection, while acting through different molecular mechanisms. It has been reported that ER α is involved in vasodilatation, attenuation of cardiac cell apoptosis and stimulation of neovascularisation. In addition, *ESR1* regulates hepatic expression of various genes involved in lipoprotein metabolism. Therefore, taken together it may be inferred that *ESR1* plays a more vital role than *ESR2* in cardiovascular maintenance [6]. The role of ER α in humans was studied for the first time in a young man with a disruptive *ESR1* mutation, who had been diagnosed with endothelial dysfunction, resulting in absence of flow-mediated endothelium-dependent vasodilatation and later premature coronary artery disease [7]. In the absence of other such cases, there was an imminent need for another approach to study the *ESR1*/CAD relationship. This led to the search for an association polymorphic variants of *ESR1* gene associated with signs and symptoms of cardiovascular pathology. *ESR1* gene single nucleotide polymorphisms, -397T>C, (rs2234693; also called *PvuII*/c.454 -397T>C/IVS1 -397T>C) and -351A>G (rs9340799; also called, *XbaI*/c.454 -351A>G/IVS1 -351A>G) are the two most studied SNPs in this gene. Both these polymorphisms are located in the first intron of *ESR1*, 397 and 351 base pairs upstream from the start of exon 2.

A large number of studies have reported an association of differential distribution of *ESR1* polymorphic variants with the diagnosis of CAD, but the results of these studies are inconsistent. Only a few studies have attempted to analyse an association between these and other polymorphic variants of the *ESR1* gene with objective measures of CAD pathology [8–10]. More experimental data is required to solve the problem of involvement of ER α in cardio protection in humans, as many important queries are still only partially answered. It appears from the conflicting observations in literature that ethnicity of the populations

may have a bearing on the association of *ESR1* polymorphism and CAD.

In this study we have attempted to elucidate the role of -397T>C and -351A>G *ESR1* polymorphisms in CAD in the north Indian population, and analyse their influence on the risk of development of CAD. In addition, we examined the association of different genotypes of these two SNPs with established risk factors of CAD viz. diabetes, hypertension etc. We hypothesized that *ESR1* gene polymorphism may influence the susceptibility to CAD through variation in ER α expression. In order to assess the veracity of this concept, we evaluated ER α mRNA expression in peripheral blood leucocytes of our cohort of CAD patients.

Methodology

Study Population

The present study was conducted on 100 north Indian consecutive patients aged 40–70 years who underwent elective coronary angiography and angioplasty (PTCA) at Gobind Ballabh Pant Hospital New Delhi, India from January 2016 to May 2017.

Inclusion Criteria

Inclusion criteria for CAD patients were presence of greater than 50% luminal stenosis in at least one major coronary artery. Inclusion criteria for age and sex matched control subjects were good general health and lack of chronic diseases including cardiovascular diseases in the medical history.

Clinical Characteristics of CAD Patients

Presence of major cardiovascular disease risk factors viz diabetes mellitus, hypertension, positive family history was noted in the cohort of CAD patients. Diabetes mellitus was defined as presence of active treatment with insulin/oral anti diabetic agent or those individuals on dietary control for diabetes for whom documentary proof of diabetic diagnosis as per American Diabetes Association criteria was confirmed. Hypertension was defined as a systolic blood pressure of ≥ 140 mm Hg and/or diastolic blood pressure of ≥ 90 mm Hg on at least 2 separate occasions or presence of antihypertensive treatment. The study was approved by Institutional Ethical Committee of Maulana Azad Medical College, New Delhi, India. Written informed consent was obtained from each patient and healthy volunteer.

Peripheral Blood Sample Collection

A peripheral blood sample (5 mL) was collected in EDTA containing vials from both patients and control subjects. Out of 5 mL of peripheral blood, 3 mL blood sample was taken for the separation of blood plasma. Peripheral blood leucocytes were isolated using RBC lysis buffer. 300 μ L of a peripheral blood sample was diluted with 900 μ L of RBC lysis buffer and incubated for 20 min at room temperature. Centrifuged the RBC lysed blood suspension at 3500 RCF for 5 min, discarded the supernatant and stored at -80°C till analysis.

ESR-1 Polymorphism Study

Restriction Fragment Length Polymorphism Polymerase Chain Reaction (PCR-RFLP)

The *ESR1* polymorphisms -397T>C (rs2234693) and -351A>G (rs9340799) were studied by PCR-RFLP technique. Briefly, genomic DNA was extracted from peripheral blood leukocytes of CAD patients and control subjects using a DNA extraction kit (Gene Aid, India) following the manufacturer's protocol. DNA concentration was determined by measuring absorbance at 260 nm. The quality and integrity of DNA was determined by the A260/280 ratio. DNA quality was also checked by ethidium bromide stained 1% agarose gel electrophoresis. The *ESR1* polymorphisms were analysed by PCR RFLP using *ESR1* gene specific forward primer (5' GATATCCAGGGT-TATGTGGCA 3') and reverse primer (5'AGGTGTT GCCTATTATATTAACCTTGA 3') for PCR amplification and digestion by restriction enzymes PvuII and XbaI for -397C>T and -351A>G polymorphisms respectively. PCR was performed in 25 μ L of total reaction volume. The PCR protocol was as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 45 s, annealing at 59.2°C for 45 s, extension at 72°C for 90 s and a final extension step at 72°C for 5 min. The PCR amplicons on 2% agarose gel electrophoresis containing ethidium bromide showed amplified fragments of 346 bp size.

This was followed by restriction digestion of the PCR amplicons at 37°C for 15 min with fast digest restriction enzymes PvuII and XbaI (Thermo Scientific, USA) for study of -397T>C and -351A>G polymorphism respectively. The C and T alleles of -397T>C polymorphism corresponded to 346 bp and $243 + 103$ bp sized fragments while in case of -351A>G polymorphism the fragment sizes for G and A alleles were 346 bp and $198 + 148$ bp respectively. The digested products were visualized on 3% agarose gel electrophoresis after staining with ethidium bromide, under UV transilluminator.

ER α mRNA Expression Study

RNA Isolation and cDNA Preparation

Total RNA, from blood plasma was extracted using Trizol method. RNA concentration was determined by measuring absorbance at 260 nm. The cDNA from isolated RNA was synthesized using a cDNA synthesis kit (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. Briefly a mixture of total RNA (100 ng) 1 μ L, 2 μ L oligo (dT) primer and 9 μ L water was incubated in thermal cycler for 5 min at 70°C and then for 10 min at 4°C . Then to this reaction mixture 5X RT buffer (4 μ L), 5 mM dNTPs (2 μ L), RNase inhibitor and reverse transcriptase 1 μ L each were added in a total volume of 20 μ L. This final reaction mixture was incubated at 42°C for 1 h. The reaction was terminated by heating the reaction mixture to 95°C for 5 min which was then chilled at 4°C .

Quantitative Real-Time PCR

Quantitative Real Time was carried out for measurement of ER α mRNA expression on a Rotor-Gene Instrument (Qiagen) using cDNA prepared as above and specific primers. *ESR 1*-F (5'-GCATTCTACAGGCCAAATTCA-3'), *ESR 1*-R (5' TCCTTGGCAGATTCCATAGC-3') and β -actin-F (5'-CCAGCACAATGAAGATCAAGATC-3'), β -actin-R (5'-ACATCTGCTGGAAGGTGGACA-3'). A reaction mixture was prepared consisting of 10 μ L SYBR Green master mix, 1 μ L cDNA 0.3 μ L forward primer (25 pmol/ μ L), 0.3 μ L reverse primer (25 pmol/ μ L) and 8.4 μ L water in a 20 μ L final reaction volume. A PCR protocol was established for simultaneous amplification of ER α mRNA and β -actin cDNA. The first segment of the amplification protocol consisted of a denaturation programme of 95°C for 10 min. The second segment consisted of three steps-denaturation (30 s at 95°C), primer annealing (30 s at 56°C) and elongation (45 s at 72°C) which was repeated for 40 cycles. The final segment consisted of a melting curve programme (ranging from 35 to 95°C).

Statistical Analysis

Compliance with Hardy–Weinberg equilibrium was ascertained in the control subjects by the Chi square test. Odds ratio was computed using codominant and dominant models for assessment of risk of development of CAD.

The Chi square/Fisher exact test were used for categorical variables and one way ANOVA/Mann–Whitney U test were used for continuous variables as appropriate. All statistical analyses were performed using Graph Pad Prism 7.03 software package. A *p* value of < 0.05 was considered as significant.

Results

Study Population

In this case control study, a total of 100 CAD patients from GB Pant hospital, New Delhi, India, 100 age and sex matched healthy controls subjects were included. The genotypic distributions for both -397T>C and -351A>G *ESR1* gene polymorphism in the control subjects were in accordance with Hardy–Weinberg equilibrium. Of the 100 CAD patients 67 were aged ≤ 55 years while 33 were of more than 55 years age. Amongst the CAD patients 81 were male and 19 were female.

ESR1-397T>C Gene Polymorphism

A representative gel electrophoretic image of *ESR1* T-397C polymorphism by PCR–RFLP with amplicon 346 bp and restriction digested products 346 bp, 243 bp, 103 bp depicted in Fig. 1.

ESR1-351A>G Gene Polymorphisms

A representative gel electrophoretic image of *ESR1*-351A>G gene polymorphism by PCR–RFLP with amplicon 346 bp and restriction digested products 346 bp, 198 bp, 148 bp depicted in Fig. 2.

Association of *ESR1* Gene -397T>C and -351A>G Gene Polymorphisms with CAD

Genotypic frequencies of *ESR1* -397T>C and -351A>G gene polymorphisms differed significantly between CAD patients and control subjects and this differential distribution reached a good statistical significance ($p < 0.0001$). Significantly, in case of the -397T>C *ESR1* SNP the frequency of mutant homozygous (CC) and heterozygous

Fig. 1 Electrophoretic gel image showing PCR–RFLP bands for *ESR1* -397T>C gene polymorphism

Fig. 2 Electrophoretic gel image showing PCR–RFLP bands for *ESR1*-351A>G gene polymorphism

(TC) genotypes were greater in CAD patients as compared to control subjects (37% and 41% vs. 30% and 19% respectively). (Table 1) The mutant C allele frequency was greater in CAD patients than in the normal controls (0.575 vs. 0.395 respectively) (Table 1). When CAD patients were segregated with respect to gender, the pattern was different in male and female patients. Importantly a significant differential genotypic distribution, with higher frequency of mutant homozygous (CC) plus heterozygous (TC) genotypes in CAD patients as compared to controls, was retained only in male subjects (60.5% vs. 39.9% respectively; $p = 0.003$) (Table 1).

Similar results were observed in case of -351A>G *ESR1* gene polymorphism with a significant difference in genotyping distribution between CAD patients and control subjects ($p < 0.001$) (Table 2). The frequencies of mutant homozygous (GG) patients and heterozygous (AG) were greater in CAD patients than in control subjects (26% and 47% vs. 20% and 23% respectively). The mutant G allele was more frequent in CAD patients in comparison to normal controls (0.495 vs. 0.315) (Table 2). As in case of -397T>C polymorphism, when the genotypic frequencies of -351A>G SNP were analysed separately in male and female subgroups, the number of mutant homozygous (GG) and heterozygous (AG) genotype came in male CAD patients was significantly higher than in corresponding control subjects (23% and 51% vs. 18.6% and 13.3% respectively; $p < 0.0001$) (Table 2).

ESR1 -397T>C (TC) and -351A>G (AG and GG) Genotypes are Associated with an Increased Risk of CAD

The risk of CAD associated with inheritance of different genotypes of *ESR1* gene -397T>C and -351A>G polymorphisms was evaluated by computation of odds ratios. In case of -397T>C polymorphism in the codominant model of analysis, the heterozygous TC genotype was observed to be significantly linked to an increase risk of CAD with

Table 1 Frequency of *ESR1* -397T>C polymorphism genotypes in CAD patients and control subjects

Subjects (No)	-397 TT genotype (%)	-397TC genotype (%)	-397 CC genotype (%)	(T) allele frequency	(C) allele frequency	Chi square	<i>p</i> value
CAD patients and control (total)							
CAD patients (100)	22 (22)	41 (41)	37 (37)	0.425	0.575	20.32	< 0.0001
Controls (100)	51 (51)	19 (19)	30 (30)	0.605	0.395		
CAD patients and control (gender specific)							
Male CAD patients (81)	32	29	20	0.5741	0.4259	11.24	0.003
Male controls (75)	45	10	20	0.6667	0.3333		
Female CAD patients (19)	5	12	2	0.2973	0.7027	5.13	0.07
Female controls (25)	6	9	10	0.42	0.58		

Table 2 Frequencies of *ESR1*-351A>G polymorphism genotypes in CAD patients and control subjects

Subjects (No)	-351AA genotype (%)	-357AG genotype (%) ~	-351GG genotype (%)	(A) allele frequency	(G) allele frequency	Chi square	<i>p</i> value
CAD patients and control (total)							
CAD patients (100)	27 (27)	47 (47)	26 (26)	0.505	0.495	27 (27)	< 0.001
Controls (100)	57 (57)	23 (23)	20 (20)	0.685	0.315		
CAD patients and control (gender specific)							
Male CAD patients (81)	21	41	19	0.5123	0.4877	31.92	< 0.0001
Male controls (75)	51	10	14	0.7467	0.2533		
Female CAD patients (19)	6	6	7	0.4737	0.5263	1.873	0.39
Female controls (25)	6	13	6	0.5	0.5		

Table 3 Risk of coronary artery disease associated with -397T>C *ESR-1* gene polymorphism genotypes

Total CAD patients					
-397T>C genotype	CAD patients	Controls	OR	95% CI	<i>p</i> value
TT	37	51	1 (Ref)	–	
TC	41	19	2.97	1.49–5.92	< 0.0001
CC	22	30	1.01	0.50–2.02	0.97
TC+CC	63	49	1.77	1.00–3.11	0.04
CAD males					
-397T>C genotype	CAD patients (male)	Controls (male)	OR	95% CI	<i>p</i> value
TT	32	45	1 (Ref)	–	
TC	29	10	4.07	1.74–9.85	0.001
CC	20	20	1.406	0.65–3.03	0.3
TC+CC	49	30	2.29	1.21–4.36	0.01

odds ratio of 2.97 (95% CI 1.492–5.92, $p < 0.0001$) (Table 3). Additionally in the dominant model inheritance of either mutant homozygous CC or heterozygous TC

genotypes, when compared to wild type TT genotype, also showed a significant association with higher CAD risk (odds ratio 1.77, 95% CI 1.007–3.11, $p = 0.04$; Table 3).

When analysed by gender, in male CAD patients versus controls, similar result were obtained, with an increased CAD risk associated with TC versus TT (odds ratio 4.07, 95% CI 1.74–9.85, $p = 0.001$; Table 3) and TC+CC versus TT genotypes (odds ratio 2.29, 95% CI 1.209–4.363, $p = 0.01$) (Table 3).

In case of -351A>G polymorphism both mutant homozygous GG and heterozygous AG genotypes were independently associated with significantly higher CAD risk in comparison to wild type AA genotype with odds ratio of 2.74 (95% CI 1.307–5.759 $p = 0.007$) and 4.31 (95% CI 2.191–8.490 $p \leq 0.0001$) respectively (Table 4).

Similarly in the dominant model inheritance of either mutant homozygous GG or heterozygous AG genotypes, when compared to wild type AA genotype, also showed a significant association with higher CAD risk (odds ratio 3.5 95% CI 1.982–6.48, $p \leq 0.0001$) (Table 4).

Analysis of male subgroup of CAD patients/control subjects revealed similar results with observation of significantly higher CAD risk in case of GG, AG and GG/AG compared to AA genotypes with odds ratio of 3.29 (95% CI 1.398–7.761, $p = 0.006$) 9.95 (95% CI 4.222–23.48, $p \leq 0.0001$) and 6.07 (95% CI 3.03–12.15, $p \leq 0.0001$) respectively (Table 4).

***ESR1*-397T>C and -351A>G Gene Polymorphisms and Their Association with Known Risk Factors of CAD Patients**

The genotypic frequency distribution observed for -397T>C *ESR1* and -351A>G gene polymorphisms in the subgroups of CAD patients were stratified by known risk factors for viz, BMI, diabetes, hypertension, and positive family history for CAD. The differential distribution of *ESR1* -397T>C reached a good statistical significant

($p = 0.01$) in case of diabetic versus non-diabetic cases. Out of 100 CAD patients, 43% were diabetic while 57% were non-diabetic. Out of 43 diabetic patients, 6 (14%) had the *ESR1* PvuII wild type TT genotype, 26 (60%) were carriers of *ESR1* PvuII TC heterozygous genotype and 11 (25.59%) had the *ESR1* PvuII CC mutant homozygous genotype whereas out of 57 non-diabetic cases TT, TC and CC genotypes were present in 15 (27.31%), 18 (37.57%) and 24 (42.1%) respectively.

Increased CAD Risk Associated with *ESR1* TC and CC Genotypes May be Due to Decrease in *ER* α mRNA Expression

The *ER* α mRNA expression in blood plasma of CAD patients was analysed and compared in the subgroups with different genotypes of *ESR1*-397T>C and -351A>G polymorphisms. In case of -397T>C polymorphism, the *ER* α mRNA expression was highest in CAD patients with wild type homozygous TT genotype ($2^{-\Delta ct} = 0.28$). A mutant 'C' allele dose dependent, significant decrease in trend in *ER* α mRNA expression was observed, with lowest expression in mutant homozygous CC genotype ($2^{-\Delta ct} = 0.09$), and intermediate expression level in heterozygous TC genotype ($2^{-\Delta ct} = 0.14$) subgroups of CAD patients. As compared to wild type TT genotype taken as reference, the fall in TC genotype (2.02 fold) and CC genotype (2.96 fold), were statistically significant ($p < 0.05$ and $p < 0.01$ respectively) (Table 5, Fig. 3). However, we did not observe any significant difference in *ER* α mRNA expression among CAD patients with heterozygous (TC) and mutant homozygous (CC) genotypes ($p > 0.05$; Fig. 3).

However, in case of -351A>G polymorphism no significant difference in CAD patients with genotype different

Table 4 Risk of coronary artery disease associated with -351A>G *ESR1* gene polymorphism genotypes

Total CAD patients					
-351A>G genotype	CAD patients	Controls	OR	95% CI	p value
AA	27	57	1 (Ref)	–	
AG	47	23	4.314	2.19–8.49	< 0.0001
GG	26	20	2.744	1.30–5.76	0.007
AG+GG	73	43	3.5	1.98–6.48	< 0.0001
CAD males					
-351A>G genotype	CAD patients (male)	Controls (male)	OR	95% CI	p value
AA	21	51	1 (Ref)	–	
AG	41	10	9.95	4.22–23.48	< 0.001
GG	19	14	3.29	1.39–7.76	0.006
GG+AG	60	24	6.07	3.03–12.15	< 0.0001

Table 5 ER α mRNA expression and *ESR1*-397T>C polymorphism genotypes

Genotype	Mean $2^{-\Delta\text{ct}} \pm \text{SD}$	Fold change	<i>p</i> value
TT	0.28 $\pm$ 0.48	2.02 fold	< 0.05
TC	0.14 $\pm$ 0.24		
TT	0.28 $\pm$ 0.48	2.96 fold	< 0.01
CC	0.09 $\pm$ 0.17

Fig. 3 *ESR1*-397T>C polymorphism and ER α mRNA expression pattern in CAD patients (**< 0.01, *< 0.05 and ns = non significant)

categories was observed. The ER α mRNA expression level in heterozygous AG ($2^{-\Delta\text{ct}} = 0.33$) and mutant homozygous GG ($2^{-\Delta\text{ct}} = 0.29$) genotypes were not significant different from that in wild type homozygous AA genotype ($2^{-\Delta\text{ct}} = 0.25$) CAD patients ($p = 0.85$ and 0.93 respectively; Table 6).

These observations suggest that increased CAD risk in *ESR1* -397T>C polymorphism TC and CC genotype carriers may be attributed to the lower *ESR1* gene expression associated with these genotypes.

Table 6 ER α mRNA expression and *ESR1*-351A>G polymorphism genotypes

Genotype	Mean $2^{-\Delta\text{ct}} \pm \text{SD}$	Fold change	<i>p</i> value
AA	0.25 $\pm$ 0.65	–	–
AG	0.33 $\pm$ 0.68	0.75	0.85
AA	0.25 $\pm$ 0.65	0.86	0.93
GG	0.29 $\pm$ 0.77		

Discussion

Coronary artery disease (CAD) is the most common cause of death worldwide [11]. Several lines of evidence suggest that endogenous estrogens have a protective effect against development of CAD. The cardioprotective actions of estrogen are mediated through their beneficial effects on the lipid profile, coagulation and/or fibrinolysis and direct effects on blood vessel walls [12]. The long term effects due to genomic actions of estrogen, lead to changes in gene expression in the vasculature and are mediated through estrogen receptor α , β , or both [13]. Both estrogen receptors are located on endothelial cells and vascular smooth muscle cells, with main protective role in cardiovascular system ascribed to estrogen receptor α (ER α) encoded by the *ESR1* gene [14].

Two tightly linked single nucleotide polymorphisms (SNPs) of the *ESR1* gene are located in intron 1, at -397 and -351 base pairs upstream of exon 2, i.e. the -397T>C (rs2234693) and -351A>G (rs 9340799) polymorphisms, which are identified by their restriction endonuclease recognition sites i.e. *PvuII* and *XbaI* respectively [15]. These 2 SNPs in the *ESR1* gene have been widely associated with the risk of cardiovascular diseases. Though *ESR1* -397T>C and -351A>G polymorphisms have been studied most extensively in different ethnic populations, the data is conflicting and there is no uniform consensus till date regarding their exact role [16]. The significance of these two SNPs in relation to CAD has not been analyzed in the Indian population.

We investigated the role of the *ESR1* gene -397T>C (rs2234693) and -351A>G (rs 9340799) polymorphisms in CAD in the north Indian population in a case control study involving 100 CAD patients and an equal number of age and sex matched healthy controls. A significant difference in the genotypic distribution of these two SNPs in CAD cases versus control subjects was observed.

In case of *ESR1* -397T>C polymorphism, the frequencies of mutant homozygous CC, heterozygous TC genotypes and the mutant 'C' allele (0.575 vs. 0.395) were significantly higher in CAD cases than in controls ($p < 0.001$). The odds ratios for the TC and TC+CC genotypes as compared to reference wild type TT genotype were 2.97 (95% CI 1.49–5.92, $p < 0.0001$) and 1.77 (95% CI 1.007–3.11, $p = 0.04$) respectively, indicating a high risk of development of CAD associated with their inheritance.

The roles of -397T>C and -351A>G SNPs in the *ESR1* gene in CAD and myocardial infarction (MI) have been examined in both prospective and retrospective association studies. Two large well designed prospective studies were published with apparent contradictory results. Shearman

et al. [17] demonstrated that in a subset of individuals ($n = 1739$) from the offspring cohort of the Framingham Heart Study [2, 18] with a follow up period of 27 years, MI attacks occurred more frequently in male subjects with -397 CC genotype than in the TT and TC genotype carriers. In contrast, Schuit et al. [19] reported in a 7 year follow-up study of a subset of individuals from the Rotterdam study including 2617 men and 3791 postmenopausal women, an increased risk of MI and ischaemic heart disease in female carriers of -397T>C and -351A>G SNPs' TT genotype and TA haplotype, while no such association was observed in men. Another large follow up study of 23–25 years in the Danish population concluded that the ER α Pvu II polymorphism was not associated with the risk of development of CAD [20].

In relation to *ESR1*-351A>G polymorphism we observed of in our cohort of CAD cases significantly higher numbers of mutant homozygous GG, heterozygous AG genotype and mutant 'G' allele (0.415 vs. 0.315) carriers ($p < 0.001$). In parallel with these results the odds ratios for GG and AG genotypes were 2.77 (95% CI 1.307–5.759, $p < 0.01$) and 4.314 (95% CI 2.191–8.490, $p < 0.0001$) respectively with reference to wild type AA genotype, indicative of greater risk of CAD in these genotype carriers. Contrary to our findings Koch et al. [21] in a large case control study observed no association of either -351A>G or -397T>C *ESR1* gene polymorphisms with risk of myocardial infarction in patients of European descent. In partial agreement with our study a case control study of *ESR1* -351A>G and -397T>C polymorphisms in Japanese population by Lu H et al. [22] reported that GG genotype and homozygosity for the CG haplotype but not the -397T>C SNP on its own, were associated with angina pectoris and MI.

A recent meta-analysis in 2015 by Jiang et al. [16] which screened 7029 CVD patients and 6901 healthy controls from 10 different case control studies, came to the conclusion that there was no significant association between *ESR1* PvuII and *XbaI* gene polymorphisms and the risk of CVD. On the contrary, another meta-analysis in 2014 of 21 case control studies by Ding J et al. [23] encompassing 9926 CAD patients and 16,710 controls revealed a significant association between *ESR1* PvuII polymorphism and CAD risk. However, when these results were stratified by region, a significant association between *ESR1* PvuII polymorphism and CAD risk was observed only in the Asian populations but not in Western populations.

When we segregated our data with respect to gender, the significant association of *ESR1* -397T>C and -351A>G SNPs with CAD observed in complete group was replicated only in male subjects. However the non-significant status of these two SNPs in females can not be asserted

because of too few number of women in our study. A gender specific effect of *ESR1* Pvu II polymorphism in males has been reported previously also by others [17, 24].

CAD is a complex disease caused by multiple genetic and environmental factors and also gene-environment interactions which may influence the role of genes. The inconsistent results in the *ESR1* -397T>C and -351A>G polymorphisms in different ethnic populations are presumably due to differences in gene expression and epigenetic effects.

The mechanism (s) by which *ESR1* gene polymorphisms may influence the occurrence/severity of CAD are far from clear. The critical importance of estrogen receptors in vessel walls in protection against CAD was initially reported by Losordo et al. [25] who showed that premenopausal women with a marked deficiency of these receptors in vessel wall had significant atherosclerotic disease. Down regulation of *ESR1* gene expression has also been implicated in the initiation and progression of atherosclerosis [26, 27].

We demonstrate in this study that *ESR1* gene PvuII (rs2234693) polymorphism is associated with a significant mutant 'C' allele dose dependent lowering of ER α mRNA expression. CAD patients carrying the TC and CC genotypes had 2.02 and 2.96 fold decreased ER α expression as compared to TT genotype carriers (p 0.001 and 0.008 respectively). This clearly indicates that *ESR1* -397T>C polymorphism affects susceptibility to CAD by a direct effect on ER α expression. In the presence of a decreased number of estrogen receptors, estrogen signalling would be less effective and result in lowering of its atheroprotective effects.

Estrogen receptor α is a ligand-activated transcription factor belonging to the nuclear hormone receptor superfamily. Transgenic mice experiments have shown that ER α causes stimulation of endothelial nitric oxide production and acceleration of reendothelialization in blood vessels [28, 29]. Pare G et al. [30] using gene knock-out mice demonstrated that ER α mediates the protective effects of estrogen against vascular injury.

Apparently contradictory to our findings, Herrington et al. [2]. earlier reported that the 'T' allele of *ESR1* -397T>C polymorphism eliminates a functional binding site for the transcription factor B-myb, which implied that this allele may result in lower ESR-1 transcription. The present study represents a direct assessment of downregulation of ER α mRNA expression in presence of the 'C' allele of *ESR1* -397T>C polymorphism, which also conferred greater susceptibility to CAD in our study population. However, the possibility that -397T>C polymorphism may be co segregating in linkage disequilibrium with another truly functional polymorphism in the *ESR1* gene can not be ruled out. In case of *ESR1* -351A>G

polymorphism, no significant change in ER α mRNA expression was seen by us in presence of AG and mutant homozygous GG genotypes.

In conclusion, this study demonstrates a significantly heightened risk of CAD associated with the inheritance of mutant genotypes of *ESR1* -397T>C and -351A>G gene polymorphisms, in the north Indian population. This is the first report of a lowered ER α expression in conjunction with the presence of mutant 'C' allele of *ESR1* -397T>C polymorphism with consequent increased CAD susceptibility.

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Compliance with Ethical Standards

Conflict of interest There is no conflict of interest to declare in this study.

Ethical Approval The study was conducted in accordance to GCP and ICMR guideline and approved by Institutional Ethical Committee of Maulana Azad Medical College, New Delhi, India. The study was approved by Institutional Ethical Committee of Maulana Azad Medical College, New Delhi, India.

Informed Consent Written informed consent was obtained from each patient and healthy volunteer.

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