

Genetic and epigenetic aspects in cardio-vascular disease and ageing

Received for publication, January 17, 2011
Accepted, September 4, 2011

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Abstract

Cardiovascular disease represents a major cause of mortality worldwide but the genetic and epigenetic mechanisms involved are not entirely known. Atherosclerosis plays a major role, often leading to ischemic heart disease and stroke. Many studies linked the evolution of atherosclerosis with genetic factors but only a small number of them are well characterized. Our study aimed to find an association between TIMP-1 (tissue inhibitors of metalloproteinases) and ESR α (estrogen receptor α) genes methylation and the polymorphisms of MTHFR (5,10-methylenetetrahydrofolate reductase) in cardio-vascular disease (CVD) and ageing. Two groups were studied: case group – 37 old patients with cardiovascular conditions and a control group – 25 young/normal subjects. The methods used for genetic and epigenetic investigations were MS-PCR (Methylation Specific Polymerase Chained Reaction) and PCR-RFLP (restriction fragment length polymorphism). In the case group, we found a higher degree of methylation of the two studied genes as well as a larger percentage of MTHFR polymorphisms. A good association between these conditions and CVD/ageing was found.

Key words: MTHFR, TIMP-1, ESR α , cardio-vascular disease

Introduction

One of the major causes of mortality in developed countries is represented by cardiovascular disease [1]. Atherosclerosis has the primary role, often leading to ischemic heart disease and stroke. Many studies linked the evolution of atherosclerosis with genetic factors, a small number of genes being well characterized [2,3]. The complexity of processes that take place in the coronary arterial wall involve inflammatory-healing response, numerous risk factors and genetic polymorphism that might increase the incidence of coronary artery disease (CAD) [4].

In 1976, Wilcken et al. indicated a possible association between plasma homocysteine level and vascular disease [5]. Since then, several studies have shown that high concentrations of plasma homocysteine may be associated with premature vascular diseases and thromboembolic vascular lesions. Therefore, plasma levels of homocysteine were considered a potential risk factor for cardiovascular disease [6-10]. The results of these studies were inconsistent regarding the entire population aspects; therefore, the mechanisms of hyperhomocysteinemia in this disease remained controversial. *MTHFR* (5,10-methylenetetrahydrofolate reductase) gene polymorphisms were established as the most important cause resulting in elevated levels of plasma homocysteine. On the other hand, in this complicated process the diet plays a major role, especially the dietary folate, vitamin B6 and B12 intake deficiency [11-13].

Homocysteine is an intermediate product in methyl group metabolism. Dietary methionine is converted to S-adenosylmethionine and then to S-adenosylhomocysteine. In reactions catalyzed by MTHFR enzyme, methyltetrahydrofolate is the donor of the methyl

group for methionine. [14,15]. As co-factor for MTHFR, riboflavin, when deficient, was also correlated with high plasma homocysteine [16,17].

A common mutation of the structural MTHFR gene is *677C3T*, resulting in alanine-to-valine substitution in the enzyme, which has a reduced activity and sensitivity to heat inactivation. The second polymorphism, *A1298C*, results in the substitution of alanine aminoacid with glutamine in C-terminal regulatory region of the protein. The enzyme activity for *A1298C* mutant homozygote gene is almost similar with the activity of the *C677T* heterozygote. The *MTHFR 677TT* genotype was associated with different types of cancer and cardiovascular disease [18,19] while *A1298C* polymorphism was associated with ovarian cancer and lymphoblastic acute leukemia [20,21].

Different stages of cardiovascular disease might be influenced by alterations in the extracellular matrix remodelling of blood vessel walls. The enzyme family that catalyses the degradation and rearrangement of extracellular matrix proteins are *matrix metalloproteinases* (MMPs) or *matrixins*, mainly produced by endothelial cells (EC), smooth muscle cells (SMC), macrophages or, in post-infarction left ventricular remodelling, by cardiomyocytes [22]. The main MMPs inhibitors are *tissue inhibitors of metalloproteinases* (TIMPs). TIMPs family consists of four members TIMP1, 2, 3, 4 [23-27]. TIMPs-1, -2, and -4 are secreted as soluble proteins, whereas TIMP-3 is associated with the matrix components as an insoluble protein.

One of the important targets of TIMP-1 is represented by MMP-9 [28]. Several studies have shown that extracellular matrix degradation by MMPs, specifically MMP-9, is involved in the pathogenesis of a wide spectrum of cardiovascular disorders, including atherosclerosis, restenosis, cardiomyopathy, congestive heart failure, myocardial infarction, and aortic aneurysm [29,30]. Local overexpression of TIMPs (TIMP-1, TIMP-2 or TIMP-3) in a human vein graft model prevented MMP-induced neointima formation [31,32]. This could be an application that offers the opportunity to prevent disease progression by locally high-level transient TIMP overexpression, within the vasculature. Even if those molecules were studied extensively, little is known about the epigenetic regulation of TIMP-1 gene.

We choose to study the methylation level of TIMP-1 gene, knowing that protein TIMP-1 exercises an inhibitory effect on most MMPs [33].

Estrogens and estrogen receptors have important physiological roles in men as well as in women. There are two known estrogen receptors: estrogen receptor α (ESR1) and estrogen receptor β (ESR2). Both receptors are expressed in a wide range of tissues, including macrophages, vascular smooth muscle, and vascular endothelial cells [34]. Estrogen receptors regulate gene expression by both estrogen-dependent and estrogen independent mechanisms that results in activation of transcription. Several studies regarding genetic association of *ESR1* gene variants in relation to coronary artery disease were realized, but limited to a few hundred individuals [35-39], to coronary artery wall atherosclerosis [40], and to variation in high-density lipoprotein, cholesterol or E-selectin levels in response to estrogen therapy [41,42].

Recent studies revealed that estrogens have a protecting role against heart disease. However, despite the fact that estrogen replacement therapy in postmenopausal women is associated with 40-50% reduction in the risk of heart disease, no effect was observed in women with known coronary artery disease [43,44]. Methylation of ER- α gene may result in an inability of SMCs to respond to protective estrogen effects leading to atherosclerotic disease.

Materials and methods

Blood samples were collected from patients with cardio-vascular conditions (n=37, aged 57-86 years old, mean = 71.5, median = 72 years old), and from healthy subjects (n=25, aged 20-53 years old, mean = 35.96, median = 35 years old).

DNA isolation. DNA was isolated from 300µl blood samples using High Pure PCR Template Preparation Kit (Roche Molecular Biochemicals, Mannheim, Germany), according to the manufacturer recommendations. DNAs concentration and purity were evaluated with NanoDrop spectrophotometer (NanoDropTechnologies, Montchanin, DE). After isolation, DNA samples were stored at -20°.

Unmethylated C residues conversion was performed with bisulphite treatment using EpiTect Bisulfite kit (Qiagen, Valencia, California, USA). Incubation of the target DNA with sodium bisulphite results in conversion of unmethylated cytosine residues into uracil, leaving the methylated cytosines unchanged. 700ng/µl of each blood isolated DNA was bisulphite treated along with positive control and negative control (CpGenome Universal Methylated DNA and CpGenome Universal Unmethylated DNA, Millipore, Billerica, MA, USA). Aliquots of bisulphite treated DNAs were stored at -80°C.

Primers. The design of selected primers discriminates between the methylated and unmethylated status of the CpG islands and do not allow misalignment. The primers were designed with the online bioinformatics tool MethPrimer. This tool convert the DNA sequence into DNA sequence bisulphite treated and shows the density of CpG islands. The DNA sequences used for the primer design were adopted for each tested gene from NCBI database (Table1). The primers were synthesized by Invitrogen Corporation (Carlsbad, CA).

Table.1 Data related to target genes and the specific primer sequences for methylated and unmethylated promoters.

Gene ID	TIMP-1	ESR- α
NCBI Genomic Reference Sequence No	NG_012533.1	NG_008493.1
Methylated primers	Forward (5'-3'): TGTTTGTTTATTTAGTTTGGTTTGC Reverse: (5'-3'): TAACGATAAATATTTAAAAAACGAT	Forward (5'-3'): TAGCGTTGTGTAGTAGTTAC Reverse: (5'-3'): AAACCTATAAAAATCAAAAAC
Amplicon size [bp]	239	150
Unmethylated primers	Forward (5'-3'): TTTATTTAGTTTGGTTTGTGG Reverse (5'-3'): TTAACAATAAATATTTAAAAACAAT	Forward (5'-3'): GTGTTGTGAGTAGTTTAGTT Reverse: (5'-3'): TCCATAAACTATAAAAATCA
Amplicon size [bp]	234	151
Function	Matrix metalloproteinases (MMPs) inhibitors	Estrogen receptor

MS-PCR (Methylation Specific PCR) consists of two different PCRs that amplified bisulphite treated DNA samples with primers for methylated and unmethylated sequences. MS-PCR was performed using Platinum Taq DNA Polymerase (1U), 1X Enzyme Buffer, 1.5mM MgCl₂, 200µM of each dNTP, 0.3 µM of each specific primer, 5µl target DNA. The final volume of PCR mix was 25µl. 35 cycles of PCR were performed for each target gene - the primers annealing conditions are presented in Table 1. The amplicons were evaluated in 2% agarose electrophoresis gel. The presence or the absence of one of the amplicons correlates with the methylation pattern of the target gene.

PCR – RFLP technique for MTHFR polymorphisms. In order to genotype MTHFR (C677T and A1298C) mutations, the methods of Frosst *et al.* [45] and Weisberg *et al.* [46] were used. The primers sequences for C677T mutation were:

5'-TGAAGGAGAAGGTGTCTGCGGGA-3' (forward) and
 5'-AGGACGGTGCGGTGAGAGTG-3' (reverse),

and the primers for A1298C polymorphism were:

5'-GGGAGGAGCTGACCAGTGACG-3' (forward) and

5'GGGGTCAGGCCAGGGGCAG-3' (reverse).

The PCR conditions were:

- *C677T* polymorphism: 30 cycles of 94°C - 45 sec., 65°C - 45 sec., 72°C -45 sec.;
- *A1298C* polymorphism: 35 cycles of 94°C- 45 sec., 65°C - 45 sec., 72°C - 45 sec.

The 198 bp (*C677T* polymorphism) and 138 bp (*A1298C* polymorphism) amplicons were subjected to enzymatic restriction with *Hinf*I and *Fnu*4 HI respectively according to the manufacturer instructions (New England Biolabs). After digestion, the samples were migrated in 3% agarose gel electrophoresis. Heterozygote (CT) presented three fragments: 198 bp, 175 bp and 23 bp. In case of *A1298C* polymorphism, heterozygote presented three fragments of 138 bp, 119 bp and 19 bp.

Statistical analysis was performed using GraphPad Instat 3.

Results and discussion

A hypermethylation of *TIMP-1* and *ESRα* genes was observed predominantly for CVD/old patients, while normal/young subjects presented mostly unmethylated genes (Figure 1).

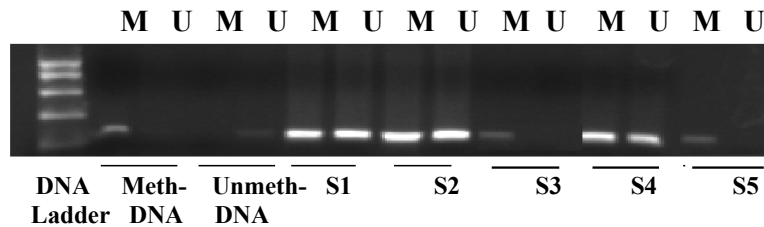


Fig. 1. Agarose gel electrophoresis for *ESRα* gene
S1, S2 and S4 samples are hemimethylated while S3, S5 samples are hypermethylated.

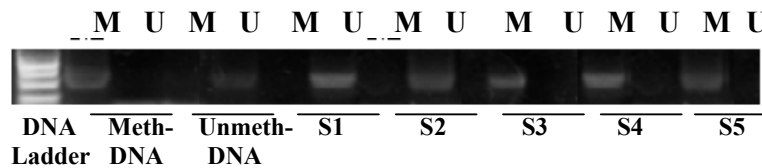


Fig. 2. Agarose gel electrophoresis for *TIMP1* gene.
S1, S2 samples are unmethylated and S3, S4 and S5 samples are hypermethylated.

ESRα and *TIMP1* genes methylation pattern observed in the two groups (CVD/old and normal/young) is presented in figure 3.

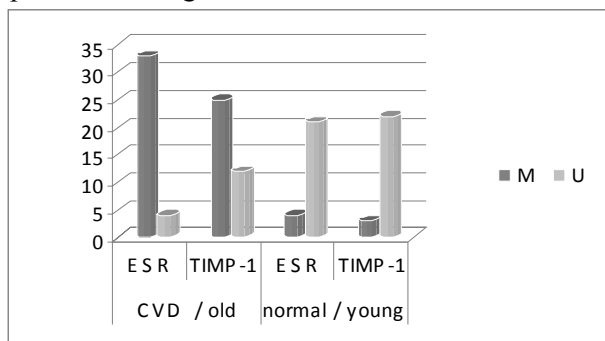


Fig. 3. Methylation status of *ESRα* and *TIMP1* genes.

In order to establish a correlation between old patients with CVD and genes hypermethylation, Fisher's exact test was performed. ESR α and TIMP1 presented a statistically extremely significant frequency of hypermethylation in old patients with CVD versus normal group (P <0.001 for each gene) (Table 2).

Specificity (percentage of healthy patients who are identified as not having the condition) and the sensitivity (predicts the capacity to identify all the patients from the CVD/old group that are presenting the disease) were calculated. The correlation was determined for ESR- α (OR=35) and for TIMP1 (OD=15.278). We found that the relative risk value is higher than 1 for hypermethylated investigated genes. This shows a good association between the epigenetic silencing of the two investigated genes and ageing and CVD.

Table 2. Fisher's exact test results for target genes.

Genes	Positive for methylation n/total cases (%)		Odd ratio	Relative risk	Specificity	Sensitivity	Positive predictive value	p-value
	Young/ normal	Old/ CVD						
ESRα	4/25(16)	33/37(89.19)	43.313	7.77	0.8919	0.84	0.84	<0.0001
Confidence interval			9.758- 192.25	3.031- 19.919	0.7461-0.9697	0.6392- 0.9546	0.6392- 0.9546	
TIMP1	3/25 (12)	25/37 (67.57)	15.278	2.713	0.8929	0.6471	0.88	<0.0001
Confidence interval			3.808- 61.288	1.677- 4.417	0.7178-0.9773	0.4647- 0.8027	0.6878- 0.9746	

The most specific (89%) and sensitive (84%) hypermethylated gene is ESR- α . TIMP1 presented a high grade of specificity (89%) but a low sensitivity (64.7%).

The MTHFR polymorphisms were studied in the two groups (CVD/old and normal/young) (Figure 4 and 5).

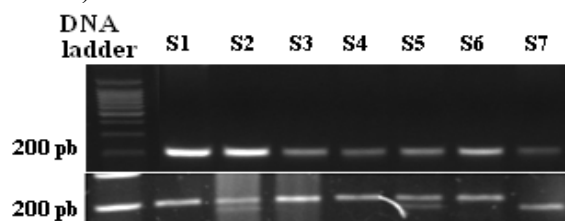


Fig. 4. MTHFR C677T polymorphism.

The upper part of Fig.4 represents the electrophoresis of the 198 bp amplicons for C677T polymorphism and the lower part shows the results of restriction reaction with *Hinf* I endonuclease. Samples S-1, 4, 6 are normal homozygote, S-2, 3, 5 are heterozygote, and S7 is mutant homozygote.

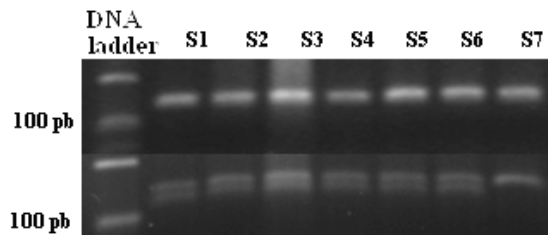


Fig. 5. MTHFR A1298C polymorphism

The samples S1-6 are heterozygote, and S7 is normal homozygote.

We found MTHFR C677T polymorphism in 59.45% of CVD/old group patients; 7/37 (18.92%) patients were mutant homozygote (TT) and 15/37 (40.54%) heterozygote (CT), while in normal/young group, only 32% presented this mutation (1/25, 4% TT and 7/25, 28% CT). Regarding A1298C mutation, we found it in 62.16% of CVD/old group. 5/37, 13.51% were TT homozygote and 18/37, 48.65% were CT heterozygote. In the normal/young group only 40% (10/25) presented this mutation, all of them being heterozygote CT. These data are represented in figure 6.

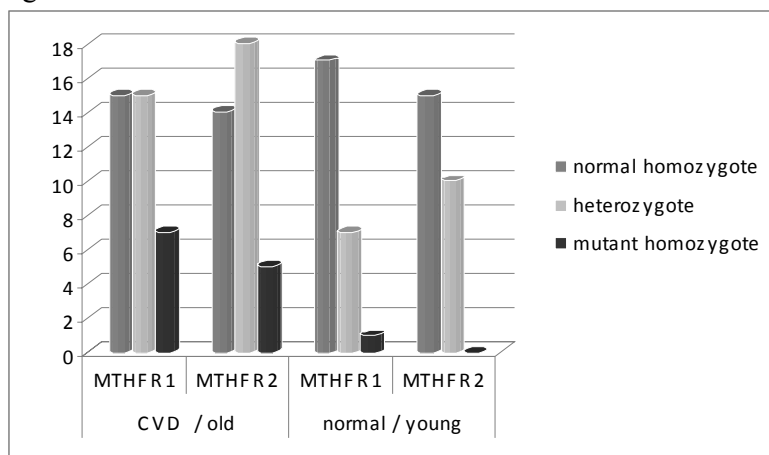


Fig. 6. MTHFR polymorphisms

The χ^2 test was performed with 1 degree of freedom, and 5% level of significance. The results support the hypothesis that both mutant alleles are susceptibility factors for CVD (C677T $\chi^2=5.409$, $p_{\text{trend}}=0.02$; A1298C $\chi^2=4.765$, $p_{\text{trend}}=0.0291$). On the other hand, heterozygote or homozygote patients with MTHFR C677T polymorphism are more likely to develop CVD than normal homozygote (OD=3.54).

In multiple systems, promoter CpG island methylation is associated with inactivation of gene transcription [47]. This process is involved in X-chromosome inactivation [48] in which promoter methylation is essential for maintaining the silenced status. CpG islands methylation in the promoter region is also involved in genomic imprinting [49]. This establishes which allele (maternal or paternal) is expressed in a given tissue. In addition, abnormal methylation of the promoter area of many genes appears to be an important feature of human neoplasia.

The methylation of ESR α and TIMP genes promoters is a hallmark for aging being involved in CVD. This study demonstrated that the TIMP1 gene promoter hypermethylation is frequent in old patients and is very rare in young people. We have to underline the fact that methylation of ESR α gene promoter is an event that appears more frequently in the middle or even earlier in life. This study established a possible link between lack of expression of the ESR α gene and atherosclerosis; some of the potential protective effects of estrogens are mediated by changes in the lipid profile, having direct effects on the vessel wall [50,51]. This observation is very important especially for menopausal women who used hormone-replacing therapy. Our data confirmed the discovery of a randomized study realized on a group of women with known coronary artery disease, study that demonstrated no benefit to hormone replacement [52]. Other study showed that in the cardiovascular system ESR α gene promoter methylation is present in a non-uniform, mosaic pattern. ESR α a gene promoter methylation can be found at variable levels in all vascular tissues including the right atrium, saphenous veins and the proximal aorta [53]. The aim of researchers is to determine the prediction potential of ESR α gene promoter hypermethylation in developing CVD and to use

demethylating agents in order to re-establish the expression of this gene in both sexes. Thus, it is more important to use demethylating treatment in combination with hormone replace therapy in women.

Regarding the role of TIMPs in cardiovascular disease, the effects of deregulation of MMPs expression are evident. Extracellular matrix degradation by MMPs, specifically MMP-9, is involved in the pathogenesis of a wide spectrum of cardiovascular disorders. On the other hand, TIMP-1 and his target MMP-9 were related to artery wall thickness. MMP-9 is known to break collagen struts and correlates with left ventricle dilatation, without necessarily affecting systolic function. Folkow *et al.* [54] showed that vascular remodelling raises blood pressure. Blood pressure, therefore, might represent a cause for left ventricle hypertrophy.

The present study showed a significant correlation ($p < 0.001$) between the promoter gene hypermethylation of investigated genes and CVD and we consider that this epigenetic modification may be a good prognostic factor.

On the other hand, our study revealed that MTHFR gene polymorphisms might be involved in a genetic predisposition of CVD acquisition. This study supports the hypothesis of Ho *et al.* [55] who examined 106 subjects with type 2 diabetes, deep vein thrombosis or coronary artery disease in order to evaluate the influence of MTHFR C677T. They found that MTHFR C677T polymorphism, vitamin B12 and triglyceride levels were the most significant factors associated with plasma homocysteine level in Chinese population. Patients with coronary heart disease presented higher plasma homocysteine levels as compared with healthy subjects and those with diabetes.

Conclusion

We found that mutant alleles of MTHFR gene are susceptibility factors for CVD and associated with ageing. Hypermethylation of the two studied genes (TIMP-1 and ESR α) can be considered as risk factors for cardiovascular diseases. New strategies for diagnosis and treatment of CVD are required in order to use them as biomarkers. On the other hand, the re-expression of TIMP1 gene may constitute not only an important biomarker, but also an important step for surgical intervention like angioplasty in order to prevent the restenosis.

Acknowledgements

This work was supported by Romanian National Research Authority Grant PN2-62057.

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