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Can APOE and MTHFR polymorphisms have an influence on the severity of cardiovascular manifestations in Italian Pseudoxanthoma elasticum affected patients?

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ABSTRACT

Background: The clinical phenotype of Pseudoxanthoma elasticum (PXE) affected patients, although progressive with age, is very heterogeneous, even in the presence of identical *ABCC6* mutations, thus suggesting the occurrence of modifier genes. Beside typical skin manifestations, the cardiovascular (CV) system, and especially the peripheral vasculature, is frequently and prematurely compromised.

Methods and results: A cohort of 119 Italian PXE patients has been characterized for apolipoprotein E (*APOE*) and methylenetetrahydrofolate reductase (*MTHFR*) gene polymorphisms by PCR. The severity of the clinical phenotype has been quantified according to the Phenodex PXE International score system. Statistical analysis (chi^2 test, odd ratio, regression analysis, analysis of variance) were done by GraphPad. Data demonstrate that the frequency of *APOE* alleles is similar in PXE patients and in healthy subjects and that the allelic variant E2 confers a protection against the age-related increase of CV manifestations. By contrast, PXE patients are characterized by high frequency of the *MTHFR*-T677T polymorphism. With age, CV manifestations in T677T, but also in C677T, patients are more severe than those associated with the C677C genotype. Interestingly, compound heterozygosity for C677T and A1298C polymorphisms is present in 70% of PXE patients.

Conclusions: PXE patients may be screened for these polymorphisms in order to support clinicians for a better management of disease-associated CV complications.

1. Introduction

Vascular calcification is a relevant clinical complication associated with aging, atherosclerosis, hypertension, chronic kidney disease and diabetes, being also correlated with increased risk of myocardial infarction. Furthermore, ectopic calcification occurs in a number of genetic diseases as in Pseudoxanthoma elasticum (PXE), a rare autosomal recessive disorder characterized by progressive mineralization of elastic fibers within soft connective tissues [1], mainly affecting skin, vascular walls and eyes [2]. In PXE, vessel alterations primarily consist of mineral plaques accumulating inside elastic fibers of medium sized arteries and veins. Therefore, the most common cardiovascular complications are: diminished or absent peripheral vascular pulses, mitral valve prolapse, arterial hypertension, angina pectoris, early intermittent claudication (often regarded as the first sign of accelerated atherosclerosis), arteriosclerosis and increased risk of myocardial and cerebral infarction [2–5]. The age-dependent progression and severity of pathologic manifestations are very heterogeneous, thus suggesting that, beside *ABCC6* causative mutations [6–8], other genes and/or polymorphisms contribute to the clinical phenotype [9–12].

In addition to changes in the expression of molecules closely associated with the calcification process [13,14], PXE subjects suffer from a condition of mild chronic oxidative stress [15–17]. It is well known that altered redox balance contributes to atherosclerosis and to vascular calcification and that several polymorphisms, as those in the apolipoprotein E (*APOE*) and 5,10 methylenetetrahydrofolate reductase (*MTHFR*) genes, are related to oxidative stress and to the development of cardiovascular complications [18].

In particular, *APOE* polymorphisms are associated with many diseases and it has been demonstrated that E2, E3 and E4 isoforms, having a different structure, exhibit a modified susceptibility to oxidation [19]. Consequently, APOE–protein interactions can be modified as well as protein function. APOE is one of the major lipid acceptor, removing cholesterol from cells and generating high density lipoprotein (HDL) particles in an isoform-dependent manner [20]. The increased risk of cardiovascular complications has been associated for instance to higher

Abbreviations: APOE, apolipoprotein E; CV, cardiovascular; Hcy, homocysteine; HDL, high density lipoproteins; MTHFR, methylenetetrahydrofolate reductase; PXE, Pseudoxanthoma elasticum.

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lipid levels in E4 carriers [21]. Moreover, it has been demonstrated that APOE isoforms, possibly depending on the number of free available –SH groups, have a different antioxidant activity (APOE2 > APOE3 > APOE4) [22,23].

Another factor significantly contributing to oxidative damage in vascular-related diseases is homocysteine (Hcy), a non-protein amino acid capable of reacting specifically, and often quantitatively, with a number of thiol-combining groups, many of which are present in proteins and in other important molecules. Irreversible homocysteinylation of long-lived proteins lead to cumulative damages and to oxidative stress [24]. Hyperhomocysteinemia can be associated to *MTHFR* gene polymorphisms or to vitamin B6, B12 or folic acid deficiency and has been involved in the pathophysiology of cancer [25–27], cardiocerebrovascular diseases, atherosclerosis [28,29], renal failure [30] and diabetic retinopathy [31]. The *MTHFR* C677T polymorphism (rs1801133) has been extensively investigated and the corresponding amino acid substitution results in a 30% to 60% decrease of the enzyme activity in heterozygotes and homozygotes, respectively [32]. A second common mutation within the *MTHFR* gene, A1298C (rs1801131), results in significantly reduced enzyme catalytic activity, even though only few studies have focused on this polymorphism [33,34]. It has been also suggested that the two polymorphisms (i.e. compound heterozygosity for C677T and A1298C) presumably act in an additive manner increasing the risk, for example, for ischemic stroke [35].

In the present study Italian PXE patients have been investigated: 1) for the frequency of *APOE* allelic variants and of *MTHFR* C677T polymorphism in order to evaluate if they are randomly present in patients or if there is an association with the disease; 2) to see if the severity of cardiovascular manifestations are influenced by these polymorphisms, thus representing additive genetic risk factors that could be counteracted by appropriate life-style.

2. Materials and methods

2.1. Patient's clinical data

Analyses were performed on 119 Pseudoxanthoma elasticum (PXE) affected patients and on 103 healthy volunteers. Since PXE is more frequently observed in females than in males [2], we have maintained a female:male ratio of 2:1 within each group.

All subjects were of Italian origin and gave informed signed consent in accordance with the guidelines of the Institutional Medical Ethical Committee and of the Helsinki Declaration of 1975 revised in 1983.

Clinical diagnosis of PXE was confirmed by the presence of calcified elastic fibers on skin biopsies and on the identification of *ABCC6* causative mutations (Supplemental Table 1) detected by already described methods [36]. Age-matched healthy individuals were selected on the basis of the absence of clinically evident pathologic conditions and of cardiovascular complications.

The severity of the pathological phenotype, as documented by clinical examinations, has been quantified according to the Phenodex score that represents, nowadays, the only published standardized system quantifying PXE clinical manifestations [37].

In particular, for the vascular system, V1 was given to weak or absent pulses, V2 to intermittent claudication and V3 to vascular occlusion or other symptoms severe enough to require surgery. For cardiac symptoms, C1 denoted ischemic changes and angina, and C2 myocardial infarction. The cardiovascular score (CV) used in the present study was the sum of V + C.

Cholesterol, high density lipoproteins (HDL), low density lipoproteins (LDL), triglycerides and homocysteine were measured in PXE patients by chemical analyses in certified laboratories under the control of the Local Sanitary Units. Patients under statin therapy were analyzed for polymorphisms, but their laboratory tests were not considered.

2.2. Identification of APOE polymorphisms

Genomic DNA was isolated from whole blood (QIAamp blood kit, Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions and stored at −80 °C until use.

Samples underwent the polymerase chain reaction (PCR) protocol described by Hixson and Vernier [38]. Briefly, DNA was amplified using oligonucleotide forward primer (5'-ACAGAATTCGCCCCGGCCTG GTACACAC-3') and reverse primer (5'-TAAGCTTGGCACGGCTGTCCAA GGA-3') (MWG-Biotech, Ebersberg, Germany) [39].

In a total volume of 30 μl, each amplification reaction contained 0.3 μg of genomic DNA from each patient, 3 μl of PCR Buffer 10x (20 mM Tris–HCl pH 7.5, 100 mM KCl, 15 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 0.5% Tween 20, 0.5% Nonidet P40, 50% glycerol) (Roche, Milan, Italy), 3 μl of each primer (MWG-Biotech), 0.6 μl of dNTPs mix (10 mM of dATP, dCTP, dGTP, dTTP) (Fermentas, Milan, Italy), 3 μl (10%) dimethyl sulfoxide (DMSO), 0.5 μl of High Fidelity *Taq* DNA polymerase corresponding to 1.73 U (Roche) plus sterile water. Samples were heated at 94 °C for 5 min for denaturation, and subjected to 30 cycles of amplification by primer annealing (60°C for 1 min), extension (70 °C for 2 min.) and denaturation (95 °C for 1 min) in a T3 Thermocycler (Biometra, Goettingen, Germany).

PCR amplification products were evaluated by agarose gel electrophoresis. Samples were digested by adding 1 μl of the restriction enzyme H*h*aI (10 U/μl, Fermentas) for 3 h at 37 °C. Each reaction mixture was loaded onto an agarose gel Metaphor (Cambrex Bio Science, Walkersville, USA) 4% in TAE 1× allowing to discriminate PCR products with small size variations. Electrophoresis was performed for 1 h and 30 min at 80 V. DNA fragments were visualized by UV illumination and the size of H*h*aI fragments was estimated by comparison with 0.1 μg of Gene Ruler 100 bp DNA Ladder Plus (Fermentas).

2.3. Identification of MTHFR polymorphisms

Detection of C677T *MTHFR* polymorphism was performed by PCR followed by HinfI restriction enzyme digestion. Briefly, we used the forward primer 5′-CCT TGA ACA GGT GGA GGC CAG-3′ and the reverse primer 5′-GCG GTG AGA GTG GGG TGG AG-3′ (Invitrogen, Monza, Italy) to amplify a 294 base pair (bp) fragment of the *MTHFR* gene. Each 25 μl PCR reaction contained 2.5 μl of 10x reaction buffer (Roche), 1.5 mmol $MgCl_2$ (Roche), 2 μl from 10 pmol of each primer, 0.2 mmol of the deoxynucleoside triphosphates, 1 U of *Taq* DNA polymerase (Roche), and 100 ng of genomic DNA template. The mixture was denatured at 95 °C for 5 min, and the PCR reaction was performed for 35 cycles in a thermocycler under the following conditions: denaturation at 95 °C for 1 min, annealing at 65 °C for 30 s, and extension at 72 °C for 1 min. The final extension cycle of 72 °C was for 7 min. The PCR products were electrophoresed on agarose gel (2%) to confirm the correct amplicon size. Restriction enzyme digestion was performed on PCR products using the HinfI restriction enzyme (Fermentas) following the supplier's protocol. After digestion, all fragments were resolved on a metaphor agarose gel (5%) (BIOSPA, Milan, Italy). A single fragment of 294 bp and two fragments of 168 and 126 bp identified the homozygous (CC) and (TT) genotype, respectively, whereas three fragments of 294, 168, and 126 bp were indicative of the heterozygous (CT) condition.

The A1298C polymorphism was detected by PCR in a total volume of 50 μl, containing: 5 μl of 10x reaction buffer (Roche), 10 pmol of the forward primer 5′-CTT TGG GGA GCT GAA GGA CTA CTA C-3′ and 10 pmol of the reverse primer 5′-CAC TTT GTG ACC ATT CCG GTT TG-3′, 10 mM of dNTP mix, 3.0 mM $MgCl_2$ (Roche) and 1 unit *Taq* polymerase (Roche) and 200 ng of genomic DNA. PCR parameters were as follows: an initial denaturation step of 2 min at 92 °C, followed by 35 cycles of 92 °C for 60 s, 51 °C for 60 s, 72 °C for 30 s and a final extension for 7 min at 72 °C to ensure a complete extension of all PCR products. PCR products were electrophoresed on an agarose gel (2%) to confirm the correct amplicon size (163 bp). The sequence analysis of this PCR fragment