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Abstract:

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To
Barry D. Kahan, PhD, MD
Editor in Chief
Transplantation Proceedings

Dear Sir:

The genetics is currently a new field incorporated for the public health and the polymorphisms story associate to the graft performance is of extreme relevance for scientific community. Thus, I am writing to submit for publication the manuscript entitled "Combination of ACE and MTHFR gene polymorphisms as determinant risk factors for Chronic Allograft Nephropathy"

Yours sincerely,

Maria Paula Sanches de Alvarenga

"COMBINATION OF ACE AND MTHFR GENES POLYMORPHISMS AS DETERMINANT RISK FACTORS FOR CHRONIC ALLOGRAFT NEPHROPATHY"

ABSTRACT

Objective: The aim of the present study was to investigate the frequency of gene Angiotensin Conversion Enzyme (ACE I/D) deletion, and Metilenetetrahydrofolate reductase (MTHFR C677T and A1298C) variants, as well to evaluated the plasma homocysteine concentrations in 217 patients submitted to renal transplantation at least 12 months after the surgery to define risk factors for chronic allograft nephropathy (CAN).

Methods: The presence of the polymorphism *ACE* deletion was assed by *polymerase chain reaction* (PCR) analysis. *MTHFR* polymorphisms were determined by PCR and RFPL (*Restriction fragment length polymorphism)* techniques. The restriction enzymes used were *Hinf I* and *Mbo II* for MTHFR variants C677T and A1298C, respectively. Plasma homocysteine concentrations were measured by liquid chromatography – tandem mass spectrometry (LS-MS/MS) and polymorphisms frequencies were investigated by polymerase chain reaction (PCR).

Results: Hyperhomocysteinemia, were more common in patients with CAN (p=0.004). No statistical significant differences were observed between the allelic and genotypic distributions of MTHFR and ACE polymorphisms. An effective risk factor was found when the polymorphisms of the *ACE* and *MTHFR* genes and hyperhomocysteinemia were associated (OR 2.51; 95% IC: 1.19 – 5.28). In conclusion our study identified that the presence of hyperhomocysteinemia in combination with unfavorable genotypes contributes to an increased risk for development of CAN.

Key words: MTHFR, ACE, renal transplantation, polymorphism, homocysteine, Chronic Allograft Nephropathy

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Running Title:

"Combination genes in chronic allograft nephropathy"

INTRODUCTION

Renal transplantation is the best therapy for patients with end-stage renal failure (ESRF) however, chronic allograft nephropathy (CAN) is the main cause of renal function loss after transplantation.[1] CAN manifests clinically as gradual reduction in renal function, accompanied by hypertension, chronic transplant glomerulopathy, tubular atrophy and interstitial fibrosis.[1] Several immunologic and non-immunologic factors have been described as responsible by development of CAN.[1] Association between genetic variants and hyperhomocysteinemia in renal allograft dysfunction has also been reported.[2Fodinger,3Pavarino-Bertelli] While there is general agreement that the mutation at nucleotide position 677 in the 5,10-methylenetetrahydrofolate reductase gene (*MTHFR* C677T) is related to hyperhomocysteinemia[2], such effects have not been demonstrated for *MTHFR* A1298C variant.[4,5] However, when combined with the C677T heterozygous polymorphism it results in a reduction of 60% of the enzymatic activity. [5] The Renin-Angiotensin System (RAS) plays an important role in the pathogenesis of fibrosis through its proinflamatory and procoagulant activities. Thus, polymorphisms of the genes that regulate activation of the RAS may be important determinants of renal transplant outcomes. RAS expression is, at least part, influenced by the polymorphisms of the angiotensin conventing enzyme (ACE).[6] The aim of this study was to evaluate the role of homocysteine (Hcy) serum levels, as well as MTHFR (A677C and A1298C) and ACE insertion/deletion (I/D) gene polymorphisms in development of CAN. In addition we tested potential interactions among these factors regarding the risk for CAN susceptibility.

SUBJECTS AND METHODS

Between Sept. 2002 to Dec. 2005 patient renal transplant recipients (n=217) were recruited at the Institute of Urology and Nephrology and included in a cross-sectional study. Inclusion criteria were transplant time of at least 12 months. The study protocol was approved by the National Ethics Committee. Patients with serum creatinine (sCr) levels persistently equal or greater than 1.5 mg/dL, creatinine clearance (LCr) less than 50 mL/min and 24-hour proteinuria equal or greater than 500 mg and a suggestive clinical follow up were diagnosed as CAN.[7] Overnight (10- to 14-hour) fasting blood samples were collected from each participant. Levels of plasma Hcy were determined by liquid chromatography – tandem mass spectrometry (LS-MS/MS).[8] Hyperhomocysteinemia was defined as

Hcy levels above 15 μmol/L.[9] Genomic DNA was extracted according to the method described by Abdel-Rahman *et al*.[10] *MTHFR* polymorphisms were determined by PCR and RFPL (*Restriction fragment length polymorphism)* techniques.[11] The restriction enzymes used were *Hinf I* and *Mbo II* for MTHFR variants C677T and A1298C, respectively. The identification of the *ACE* gene deletion polymorphism (D14091-14378) was performed by PCR analysis.[12] For *ACE* genotyping, PCR products were electrophoresed on 2% agarose gel and alleles were visualized as 190bp (D) or 490bp (I) fragments. When a DD genotype was found, the DNA was amplified using insertion-specific primers to confirm the absence of the I allele. Descriptive statistics analyses included mean values ± standard deviations (SD) for continuous data and percentages for categorical data. The prevalence of the different genotypes was compared using the Fisher Exact test. Differences in Hcy concentrations were analyzed by the Mann-Whitney test. Binary logistic regression was used to assess the risk of development CAN in hyperhomocysteinemia and in those genotypic combinations in which odds ratios (ORs) and 95% confidence limits (CL) were estimated. A p-value of less than 0.05 was considered to indicate statistical significance.

RESULTS

A total of 217 renal transplant recipients (80 women, 137 men; mean age 41.0 ± SD 13.6 years; time since transplantation 5.8 ± SD 3.5 years) were analyzed. The mean values for sCr (2.5mg/dL ± SD 1.3), LCr (36.4mL/min ± SD 18.4) and 24-hour proteinuria (639.4mg ± SD 882.3) in the CAN Group did not demonstrate significant differences with compared to FRN group (mean values = 1.1mg/dL ± SD 0.3, 68.6mL/min ± SD 23.1, and 218.5mg ± SD 439.7, respectively). Hyperhomocysteinemia, was observed in 175 (80.6%) of the 217 patients included in the study and statistical analysis demonstrated a significant difference between NRF and CAN groups (p=0.004). Additionally, the mean serum Hcy level was significantly higher in patients with CAN than in patients with NRF (31.5 μmol/L [6.4 – 183.0] *versus* 23.4 μmol/L [3.5 – 170.0]; p<0.0001). The most common genotypes observed in both groups were 677CT/1298AA/ID (15.2%) and 677CT/1298AC/ID (12.0%). No statistical significant differences were observed between the allelic and genotypic distributions of *MTHFR* and *ACE* polymorphisms. However, the association of the three studied polymorphisms,

677/1298 MTHFR and ACE, showed a positive tendency for the CAN group (p=0.074). Hyperhomocysteinemia associated to presence of at least one *MTHFR* allelic variant, (677CT/1298AC; 677CT/1298CC; 677TT/1298AC genotypes), was significantly more common in the group with CAN (p=0.023) and a significant risk of this association was observed for the development of CAN (OR= 2.33; 95% IC: 1.09-4.13). In addition to these factors, the ACE (I/D) polymorphism significantly increased the risk for CAN (OR: 2.51 95% IC: 1.19-5.28).

DISCUSSION

It has been shown that high levels of homocysteine induces sustained injury of arterial endothelial cells and acceleration in the development of thrombosis and atherosclerosis. The mechanism by which homocysteine might cause vascular damage is unclear. Experimental evidence suggests that homocysteine promotes atherogenesis by facilitating oxidative arterial injury, damaging the vascular matrix, and augmenting the proliferation of vascular smooth muscles.[13] Our study evidenced elevated levels of homocysteine in 175 patients (80.6%), agreeing with Marcucci *et al.,* 2001[13] in a study of transplanted patients (90%), and support the hypothesis that higher Hcy levels has influence in vascular renal disease. Abnormal plasma Hcy concentrations can result from genetic defects, abnormal vitamin nutritional status, or both.[2] Methylenetetrahydrofolate reductase (MTHFR) plays a key role in Hcy metabolism.[4] Polymorphisms of the *MTHFR* gene have been associated to the development of CAN in patients submitted to renal transplantation and several studies identified the effects of different *MTHFR* genotypes on the Hcy metabolism in renal transplantation recipients. [2,3,4,13] In our study the allelic frequency of the *MTHFR* gene polymorphisms in renal transplant recipients (677T 0.39 and 1298C 0.30 CAN; and 677T 0.38 and 1298C 0.25 NFR) was comparable to those found in other investigations[2,4], and did not differ from that in the healthy population. However, the presence of allelic variants for both of the significant factor for development of CAN. The I/D polymorphism of the ACE gene, been suggested as an additional risk factor for the progression of chronic renal insufficiency in ESRF and IgA nephropathy, the I/D polymorphism, with the ACE-DD genotype associated to the progression.[6,14] But the data available in this area is still conflicting.[15,16,17] In our study an influence of the ACE polymorphic variant in the development of CAN was not established. An effective risk factor was found when polymorphisms of the *ACE* and *MTHFR* genes

and hyperhomocysteinemia were associated. In our knowledge, this is the first study to establish that this combination may increase the susceptibility to CAN.

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