

## CLINICAL SCIENCE

# Genotype analysis of the human endostatin variant p.D104N in benign and malignant adrenocortical tumors

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**OBJECTIVE:** Endostatin is a potent endogenous inhibitor of angiogenesis. It is derived from the proteolytic cleavage of collagen XVIII, which is encoded by the *COL18A1* gene. A polymorphic *COL18A1* allele encoding the functional polymorphism p.D104N impairs the activity of endostatin, resulting in a decreased ability to inhibit angiogenesis. This polymorphism has been previously analyzed in many types of cancer and has been considered a phenotype modulator in some benign and malignant tumors. However, these data are controversial, and different results have been reported for the same tumor types, such as prostate and breast cancer. The purpose of this study was to genotype the p.D104N variant in a cohort of pediatric and adult patients with adrenocortical tumors and to determine its possible association with the biological behavior of adrenocortical tumors.

**METHODS:** DNA samples were obtained from 38 pediatric and 56 adult patients (0.6–75 yrs) with adrenocortical tumors. The DNA samples were obtained from peripheral blood, frozen tissue or paraffin-embedded tumor blocks when blood samples or fresh frozen tissue samples were unavailable. Restriction fragment length polymorphism analysis was used to genotype the patients and 150 controls. The potential associations of the p.D104N polymorphism with clinical and histopathological features and oncologic outcome (age of onset, tumor size, malignant tumor behavior, and clinical syndrome) were analyzed.

**RESULTS:** Both the patient group and the control group were in Hardy–Weinberg equilibrium. The frequencies of the p.D104N polymorphism in the patient group were 81.9% (DD), 15.9% (DN) and 2.2% (NN). In the controls, these frequencies were 80.6%, 17.3% and 2.0%, respectively. We did not observe any association of this variant with clinical or histopathological features or oncologic outcome in our cohort of pediatric and adult patients with adrenocortical tumors.

**KEYWORDS:** Endostatin; Angiogenesis; p.D104N polymorphism; Adrenocortical tumor.

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## INTRODUCTION

Collagen XVIII/endostatin is a heparan sulfate proteoglycan component of nearly all epithelial and endothelial basement membranes (1). The gene *COL18A1* is located on chromosome 21 at 21q22.3 and contains 10 collagenous domains that are interrupted and flanked by non-collagenous domains (2). A proteolytic fragment produced

by the cleavage of the C-terminal non-collagenous domain (NC1) is known as endostatin and has been shown to exhibit anti-angiogenic activity *in vitro* and *in vivo* (3). As an angiogenic inhibitor, endostatin prevents tumor growth and expansion by controlling the formation of new blood vessels (4). The observation that individuals with trisomy 21 (Down's syndrome) rarely develop solid tumors suggests the presence of an endogenous angiogenesis inhibitor encoded by a gene on chromosome 21 (5). A single nucleotide polymorphism c.4309G>A (p.D104N) was identified in the endostatin domain of *COL18A1* (6). Lughetti et al. reported that, relative to control individuals, patients who are heterozygous for this polymorphism (DN) had a 2.5-fold greater chance of developing solid tumors, such as prostate cancer (7). This finding likely

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No potential conflict of interest was reported.

indicates that this polymorphism results in a less active protein and that individuals who carry one or more mutant alleles at this locus are more prone to developing aggressive prostate cancer (7). In contrast, Macpherson et al. observed no significant difference in the normal homozygous (DD), heterozygous (DN), and mutant homozygous (NN) frequencies between androgen-independent prostate cancer patients and control individuals (8).

During embryonic development and postnatal growth and repair, angiogenesis or neovascularization from the preexisting vasculature is crucial (9). The formation of new blood vessels is a complex multistage process that involves the proteolytic degradation of the basement membrane, the loss of endothelial cells into the surrounding stroma, and finally, the re-adhesion of endothelial cells to form new capillary tubes (9). The growth of tumors is associated with increased angiogenesis, during which the formation of new blood vessels is a fundamental step in tumor development and expansion. In addition, neovascularization is a critical component of tumor metastasis and disease progression (10). This connection between angiogenesis and tumor progression has been documented in clinical studies, which have demonstrated a correlation between unbalanced angiogenic factors and metastatic tumors with poor survival rates (11). The normal (i.e., non-tumorous) adrenal gland is a highly vascularized tissue that is centripetally irrigated by a network of fenestrated capillaries (12). Whereas adrenocortical tumors and the normal adrenal cortex have a similar vascular pattern (13), adrenocortical carcinomas display a disorganized vasculature, with large vessels interspersed with irregular networks of microcapillaries (13). As endostatin is involved in angiogenesis, we hypothesized that the presence of the D104N polymorphism may influence the outcome of adrenocortical cancer.

**OBJECTIVE**

The aim of this study was to analyze the frequency of the DD (normal homozygous), DN (heterozygous), and NN (mutant homozygous) genotypes of the p.D104N polymorphism in patients with adrenocortical tumors and to identify any associations with clinical and biological features. The cohort consisted of pediatric and adult patients with adrenocortical tumors (benign and malignant) who were seen at the Hospital das Clínicas, University of São Paulo, Brazil, from 1990 to 2010.

**PATIENTS AND METHODS**

**Subjects**

DNA samples were obtained from different tissues depending on the viability of the samples. Peripheral blood (n = 17), fresh frozen tissue (n = 70), and paraffin embedded tissue samples (n = 7) were collected from 94 patients with adrenocortical tumors. Thirty-eight patients were pediatric (1 to 17 yrs of age; 10 carcinomas, 7 with a poor outcome), and fifty-six were adults (18 to 70 yrs of age; 25 carcinomas, 17 with a poor outcome).

The clinical data are presented in Table 1. All of the cases were diagnosed between 1990 and 2010. In addition, 300 alleles from 150 healthy volunteers were analyzed to determine the frequency of the D104N endostatin polymorphism.

**Table 1 - Clinical data from 94 patients with adrenocortical tumors.**

Characteristic	Pediatric group	Adult group
Number of patients	38	56
Median age in years (range)	2.5 (0.8–18)	35.28 (18–74.8)
Gender	10M/28F	6M/50F
Hormone-secreting tumors	38	47
Tumor size (cm)	5.6 (1.5–20)	4.5 (2–20)
Weiss score		
0–2	12	29
3–5	15	14
6–9	11	13
MacFarlane stage		
I–II	32	34
III–IV	6	22
Specific death	7	16
Time to death, months (range)	13.7 (7–16)	13.6 (8–35.4)
Follow-up, months (range)	66 (1–167)	44.6 (1–203)

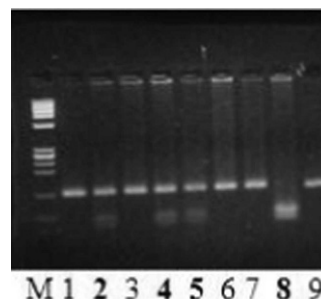
\*A Weiss score ≥3 and a MacFarlane stage ≥III were considered adrenocortical carcinoma in the pediatric group. A Weiss score ≥3 was considered adrenocortical carcinoma in the adult group.

**DNA amplification and restricted fragment length polymorphism analysis**

A fragment from exon 42 of the COL18A1 gene was amplified using PCR with the following primers: 5'-ACA AAC ACC CAC ACC CAT C-3' and 5'-GGG CTC CTA TCT GCA GTT TC-3'. For paraffin-embedded tumor blocks, the following primers were used: 5'-GCT GGG AGG CTC TGT TCT CAG-3' 5'-TAG GTT CCC AGT GCC GTG TGA-3'. When the DNA was obtained from fresh tissue or leukocytes, we were able to amplify a 261 bp fragment, but when the DNA was obtained from paraffin-embedded tumor blocks, new primers were designed to amplify a 119 bp fragment. PCR amplification was performed using a previously described protocol (14).

Both of the fragments (261 and 119 bp) were subjected to RFLP analysis using the restriction enzyme MseI, and the fragments were separated by electrophoresis in a 2.0% agarose gel.

The 261 and 119 bp fragments were subjected to RFLP analysis using the restriction enzyme MseI, and the fragments were separated by electrophoresis in a 2.0% agarose gel (Figure 1).



**Figure 1 - Electrophoresis of PCR and RFLP – The lines 2, 4 and 5 indicate the heterozygous pattern. The line 8 represents the homozygous pattern and the M corresponds to the molecular weight marker 0x.**

## Statistical analyses

Descriptive analyses were performed to determine the frequencies and distributions of the demographic variables. The genotype frequencies in the adrenocortical tumor patients and control individuals were compared using contingency table analyses and the chi-square or Fisher's exact test, when necessary. The odds ratio and the corresponding confidence intervals were estimated using standard methods. Statistical analyses were performed using STATA 9.0 statistical software. The Hardy-Weinberg equilibrium was tested with the chi-square test to determine the goodness-to-fit (one degree of freedom).

## RESULTS

The frequencies of the alleles in both the patients and controls were in Hardy-Weinberg equilibrium.

Of the 94 patients, 15 were heterozygous for the p.D104N polymorphism (DN), and 2 were homozygous for the p.D104N polymorphism (NN). Of the 150 controls, 26 were heterozygous for the p.D104N polymorphism, and 3 were homozygous for the mutant allele. The genotype frequency of this polymorphism did not differ between the patients (17%) and controls (19%) ( $p=0.82$ ).

Of the 17 patients carrying the mutant allele, 14 were adults; 8 of these adult patients were diagnosed with adenoma, and 6 were diagnosed with carcinoma. All of these patients were heterozygous (DN) for the polymorphism ( $p=0.50$ ). The three remaining patients carrying the mutant allele were pediatric patients, and all were diagnosed with a benign adrenocortical tumor. Two of these pediatric patients were homozygous (NN) for the mutant allele, and one was heterozygous (DN) ( $p=0.38$ ). No statistical difference was observed among the genotypes frequencies (DD, DN, and NN) in this cohort with respect to the clinical data, including the presence of Cushing's syndrome ( $p=0.76$ ), the presence of virilization ( $p=0.07$ ), tumor weight or size ( $p=0.35$ ), Weiss score ( $p=0.3$ ), metastasis occurrence ( $p=0.43$ ), disease recurrence ( $p=0.51$ ), and patient mortality ( $p=0.8$ ).

## DISCUSSION

Following the observation that individuals with trisomy 21 (Down's syndrome) rarely develop solid tumors, Folkman and Kalluri proposed that an endogenous angiogenesis inhibitor is likely encoded by a gene on chromosome 21 (5). Endostatin was first isolated from the conditioned media of a non-metastatic murine hemangioendothelioma cell line, EOMA. EOMA cell-conditioned media inhibited the proliferation of FGF-2-stimulated bovine capillary endothelial cells (15).

Preclinical experiments in which endostatin is exogenously administered to cancer patients have been used to evaluate its anti-tumor activity and to determine whether its endogenous anti-angiogenic effect controls the formation of new blood vessels, which is a necessary event for tumor metastasis (16).

A non-synonymous polymorphism (p.D104N) in exon 42 of *COL18A1*, which encodes endostatin, was described by Yugetti et al. The presence of this mutation leads to decreased activity of the protein, which may 1) interfere with the anti-angiogenic role of endostatin and 2) be associated with the progression of solid tumors, such as breast cancer and prostate cancer (7,17). This particular

polymorphism has also been evaluated in other types of cancers, such as leukemia (18). However, there have been contradictory reports regarding the association between this polymorphism and prostate tumors and breast tumors when analyzed in different cohorts (7,18).

Adrenocortical tumors are highly vascularized tumors; we therefore hypothesized that a functional polymorphism located in the endostatin-coding region of *COL18A1* may be associated with the poor behavior of the tumors, leading to early metastasis and death.

In the present study, we identified no association between the p.D104N endostatin polymorphism and tumor aggressiveness or other clinical features in pediatric and adult patients. The lack of an association observed in our adrenocortical cancer population has also been observed in other tumors types. Similar studies of patients with leukemia and multiple myeloma revealed no association between the endostatin p.D104N polymorphism and disease outcome (18,19). The discrepancy between these results may be due to the limited size of the studied population. For example, studies performed by Iughetti et al. and Lourenço et al. (20) revealed a high risk of sporadic breast cancer in individuals homozygous (NN) for the p.D104N polymorphism. However, these studies examined a small number of patients, and the allelic frequencies were not in Hardy-Weinberg equilibrium ( $\chi^2=22.87$ ,  $p<0.001$ ). Alternatively the different results could also be due to disease heterogeneity. Other candidate genes should be investigated with respect to their involvement in angiogenesis.

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## AUTHOR CONTRIBUTIONS

Mariani BMP was responsible for the PCR and RFLP statistical analyses. Trarback EB was responsible for the primer design and optimization. Ribeiro TC was responsible for the DNA extraction. Pereira MAA was responsible for the patient assistance. Mendonca BB supported the work, suggested the study and was also responsible for the patient assistance. Fragoço MCBV was responsible for the patient assistance and work support.

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