A Polymorphism in Endostatin, an Angiogenesis Inhibitor, Predisposes for the Development of Prostatic Adenocarcinoma


Abstract

We have performed association studies between a novel coding single nucleotide polymorphism (D104N) in endostatin, one of the most potent inhibitors of angiogenesis, and prostate cancer. We observed that heterozygous N104 individuals have a 2.5 times increased chance of developing prostate cancer as compared with homozygous D104 subjects (odds ratio, 2.4; 95% confidence interval, 1.4–4.16). Modeling of the endostatin mutant showed that the N104 protein is stable. These results together with the observation that residue 104 is evolutionary conserved lead us to propose that: (a) the DNA segment containing this residue might contain a novel interaction site to a yet unknown receptor; and (b) the presence of N104 impairs the function of endostatin.

Introduction

Prostate cancer is the most common male malignancy and the second leading cause of cancer-related deaths in American men (1). It has been estimated that although 42% of males have prostate carcinoma identified by postmortem examination, only 9.5% will have a clinical diagnosis in their lifetime, and only 2.9% actually will die of the disease (2). Hence, the development of prognostic tests is essential to identify those patients who would benefit from more vigilant surveillance. Although the majority of prostate cancer cases are sporadic, it has long been recognized that familial clustering exists, with an increased relative risk of affected families (3). Segregation analysis of prostate cancer suggests the presence of at least one major susceptibility locus that may account for up to 10% of all cases (3). Indeed, at least six putative prostate cancer susceptibility loci (five on autosomal chromosomes and one on the X chromosome) have already been mapped through linkage analysis (4, 5). In addition to these putative major susceptibility genes, it is believed that alterations of other genes could be associated with the risk and/or progression of this type of tumor, including both sporadic and familial cases (6).

Angiogenesis, or the formation of new blood vessels from preexisting endothelium, is a fundamental step in tumor progression and metastasis (7, 8). A wide range of both stimulatory and inhibitory molecules mediates the sequential steps involved in angiogenesis. Endostatin, a $M_r$ 20,000 cleavage product of the COOH-terminal globular domain NC1 of collagen XVIII, the encoding region for endostatin (Table 1). We hypothesized that the presence of an asparagine instead of aspartic acid at this position may lead to an impaired function of endostatin and could therefore be associated with the progression or aggressiveness of solid tumors. Our analysis of this cSNP in 181 prostate cancer patients and 198 control individuals suggests that this change is predisposing to the development of malignant tumors of the prostate.

Materials and Methods

Patients and Controls. A total of 181 prostate cancer cases (61 Caucasians, 11 blacks, and 109 unclassified) of mean age 65.8 years (SD, 8.1 years) comprises the case group, 50 from the Hospital do Câncer and 131 from the Hospital Oswaldo Cruz, both situated within the city of São Paulo, Brazil. All cases had fully documented histopathological diagnosis and were graded according to Gleason’s system. A total of 198 non-cancer individuals (106 Caucasians, 83 blacks, and 9 unclassified) of mean age 52.7 years (SD, 14.1 years), who were relatives of patients with neuromuscular disorders and with negative history for prostate cancer, were considered as controls.

DNA was obtained from blood (in 66 cases and all control individuals) or from paraffin-embedded tissues (115 cases). Standard protocols for DNA extraction from each of these tissues were used.

Genotype Analysis. The missense change D104N (amino acid position in endostatin, which corresponds to amino acid position 1437 and nucleotide 4349G→A of the cDNA medium form of collagen XVIII) leads to the creation of a restriction site for MseI. This change was analyzed through the amplification of a 169-bp fragment using the primers 5′-ccagtttctctctgagac-3′ and 5′-ctctcaggtgcctcagc-ag-3′, followed by restriction endonuclease digestion

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The abbreviations used are: cSNP, coding single nucleotide polymorphism; OR, odds ratio; CI, confidence interval.
DNA, PfxTaq DNA polymerase (Life Technologies, Inc.; 0.2 unit), and 250 μM of each deoxynucleotide triphosphate, at 94°C for 40 s, 57°C for 40 s, and 72°C for 1 min. The restricted digested PCR products were separated on 8% polyacrylamide gels stained with silver nitrate by standard procedures. In the presence of the D104N mutation was also performed in DNA samples asparagine.

**Results and Discussion**

Association between an Endostatin Polymorphism (D104N) and Prostate Cancer. The distribution of the genotypes for the polymorphism D104N is reported for controls and prostate cancer patients in Table 2. We did not observe any significant difference in the frequency of this polymorphism between Caucasians and blacks in controls (P = 0.13) or in patients (P = 0.16), and this variable was not considered in the majority of statistical analysis. In addition, we found very similar genotypic frequencies for this polymorphic system in the patients ascertained in the two hospitals (33% in patients from Hospital do Câncer and 22% in patients from Hospital Oswaldo Cruz). All patients were therefore considered as a unique group for analysis.

Both the patients’ and controls’ samples are in Hardy-Weinberg equilibrium (χ² = 1.82, P = 0.41; χ² = 0.82; P = 0.66, respectively), but the frequency of heterozygotes for the D104N polymorphism was significantly higher in patients as compared with the control group (χ² = 10.23; P = 0.0014; Table 2).

These findings support for the first time a potential association between a polymorphism in endostatin, one of the most potent angiogenesis inhibitors, and prostate cancer. Our results predict that individuals heterozygous for N104 have a 2.5 times greater chance of developing prostate cancer (OR, 2.4; 95% CI, 1.4–4.2). Association studies have been biased by potential stratification. In our sample, we

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Subjects with racial classification</th>
<th>Caucasians (n/%)</th>
<th>Blacks (n/%)</th>
<th>Total sample n (%)</th>
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<tr>
<td>Controls</td>
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<tr>
<td>DD</td>
<td>89 (84%)</td>
<td>76 (92.6%)</td>
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<tr>
<td>DN</td>
<td>17 (16%)</td>
<td>7 (8.4%)</td>
<td>24 (12.1%)</td>
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<tr>
<td>Total</td>
<td>106 (100%)</td>
<td>83 (100%)</td>
<td>189 (100%)</td>
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</tr>
<tr>
<td>Patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DD</td>
<td>43 (70.5%)</td>
<td>5 (45%)</td>
<td>48 (74.5%)</td>
<td></td>
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<tr>
<td>DN</td>
<td>18 (29.5%)</td>
<td>6 (55%)</td>
<td>24 (24.9%)</td>
<td></td>
</tr>
<tr>
<td>NN</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>61 (100%)</td>
<td>11 (100%)</td>
<td>72 (100%)</td>
<td></td>
</tr>
</tbody>
</table>

with Msel. The PCR reaction consisted of 32 cycles of amplification and used the primers described above at concentrations of 4 μM, 100 ng of genomic DNA, PfxTaq DNA polymerase (Life Technologies, Inc.; 0.2 unit), and 250 μM of each deoxynucleotide triphosphate, at 94°C for 40 s, 57°C for 40 s, and 72°C for 1 min. The restricted digested PCR products were separated on 8% polyacrylamide gels stained with silver nitrate by standard procedures. In the presence of the mutation, two fragments of 101 and 68 bp were generated. All samples were done in duplicate to ensure genotyping accuracy. Analysis for the presence of the D104N mutation was also performed in DNA samples extracted from two different regions (a normal and a tumor one) of the paraffin block of 20 patients chosen randomly.

**ELISA.** ELISA analysis was performed in serum from 26 control individuals (13 heterozygous for the polymorphism D104N and 13 homozygous for the most common allele). Blood samples were collected in EDTA-containing tubes, and serum was separated and stored at −70°C for future use. ELISA for serum endostatin was performed using a commercially available assay (Accucyte; Cytimmune Sciences, Inc., College Park, MD), according to the manufacturer’s instructions for usage. All measurements were performed in duplicate to ensure the accuracy of the data collected. The kit used has a sensitivity of 2 ng/ml, and typical interassay and intrassay variances were 10% or less.

**Molecular Modeling and Mutant Structure Analysis.** The human endostatin structure was obtained from the Protein Data Bank accession code 1KOE and analyzed using the programs GRASP (16), Swiss PDB Viewer (17), O (18), and Insight-II/Discover (19). The observed mutation was created by Insight-II and optimized by molecular energy minimization fixing all atoms except the side chains of D104N and its neighbors S102, K106, and T113. The structures of mouse endostatin derived from collagen XVIII (PDB accession code 1KOE) and mouse endostatin derived from collagen XV (PDB accession code 1DY2) were also used for comparison.

**Statistical Analysis.** Gene and genotype frequencies in affected and normal individuals were compared by contingency table analysis using both χ² statistics and Fisher’s exact test. The level of significance considered was 5% and 1 degree of freedom for χ² analysis. Hardy-Weinberg equilibrium was tested with the χ² statistic for the goodness-of-fit (1 degree of freedom). The OR and its CI were estimated by standard methods (20).

![Fig. 1. Superposition between the modeled structures of human endostatin XVIII and the mutant D104N. The residues labeled in this figure were the only not fixed during the energy minimization protocol. The residue K106(mod) corresponds to the final conformation in the structure modeled (differs from the deposited structure), whereas K106(mat) corresponds to the final conformation calculated for the mutated D104N structure.](image-url)
were able to obtain ethnic origin of just a small number of patients. However, we observed that the Caucasian cases have a significantly higher carrier frequency than the Caucasian controls \((P = 0.0488;\) OR, 2.19; 95% CI, 1.0–4.6). The same was observed for the black population \((P = 0.0007;\) OR, 13.03; 95% CI, 3.3–50.8). Therefore, even if the OR derived from the combined analysis has been slightly biased by potential stratification, the result is significant in both identifiable subsets themselves, confirming the results obtained with combined data. We did not observe any association between the presence of this mutation and Gleason score or age at diagnosis (Table 3), suggesting that this alteration is more related to the genesis of the tumor than to its aggressiveness. Recently, Musso et al. \((21)\) reported that endogenous collagen XVIII levels in recurrent hepatocellular carcinomas were 2.2-fold lower than in those hepatocellular carcinomas that did not recur, a finding which is in agreement with our hypothesis that individuals with higher levels of endostatin might be less prone to develop solid tumors.

**D104N Polymorphism Possible Leads to an Impaired Endostatin Function.** We have aligned the sequences of both human and mouse endostatin XVIII as well as the endostatin-like molecule that is produced from the NC1 domain of collagen XV, which presents 61% sequence identity with that of collagen XVIII \((22)\). This analysis showed that the aspartic acid at position 104 is conserved in all cases. In all of the three crystal structures, this residue is located at the surface of the molecule, partially buried through a hydrogen bond between OD1 (oxygen delta 1) and an internal serine residue (S102 in the human endostatin XVIII). The atom OD2 (oxygen delta 2) is surrounded by positively charged residues, and in the high-resolution mouse structures, it is interacting through a strong salt bridge to an arginine residue. In the human endostatin, however, this arginine corresponds to K106, the NZ (nitrogen zeta) of which is in close proximity to the OD2 of D104.

The modeling of the D104N mutant could encompass two possible arrangements for the asparagine side chain. In one configuration, the \(-\text{NH}_2\) group would occupy the buried OD1 position, necessarily interacting with S102, which is unlikely to happen because the residue S102 is acting as a proton donor to D104 and a proton acceptor for the main chain \(-\text{NH}_2\) group of K106. Therefore, the second configuration is favored, with the \(-\text{NH}_2\) group of the mutant N104 occupying the OD2 position of D104. Starting from a modeled mutant structure based on the second configuration, the molecular energy minimization shows little rearrangement of the surrounding residues to accommodate the new \(-\text{NH}_2\) group of N104. On the other hand, the same procedure applied to the human structure resulted in a different conformation for K106, in terms of the distance to a salt-bridge to D104 (Fig. 1). Fig. 2 represents the electrostatic potential at the surface of both wild-type and the mutated structures, showing the altered charge distribution surrounding the D104N mutation.

The structure modeling analysis thus suggests that the human mutated D104N protein is stable, a result further supported by the finding that endostatin XVIII serum levels were similar both in carriers and non-carriers of this mutation \((17.38 \pm 5.55 \text{ ng/ml}; 17.17 \geq 5.99 \text{ ng/ml})\).

Recent studies have shown that inhibition of endothelial cell proliferation and migration of cultured endothelial cells might occur through the binding of an endostatin epitope of two clusters of arginine to heparin/heparan sulfate when angiogenesis is induced by FGF-2 \((23)\). This heparin binding epitope does not include D104; however, it is possible that this amino acid and its surrounding residues are presented for the interaction with a yet undetermined target receptor. The existence of another interaction site in endostatin is also supported by the observation that inhibition of vascular endothelial growth factor-induced migration of endothelial cells is not dependent on its heparin-binding epitope. Thus, we hypothesize that the D104N mutation might decrease the ability of endostatin to bind other molecules, thereby impairing its ability to inhibit angiogenesis. The amino acid D104 is conserved in both humans and mice, and it is also conserved in endostatins from both collagen XV and XVIII, implying that this residue is in a functionally important region of endostatin, with a common role for both angiogenesis inhibitors. It will be important to perform functional analyses to test the above hypothesis. The confirmation of the association of N104 with prostate cancer in other populations will also be important to support the possible screening for this mutation as a predictive test for prostate cancer.

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**References**


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