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Endostatin polymorphism 4349G/A(D104N) is not associated with aggressiveness of disease in postate cancer[1]

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Abstract. Endostatin is an important inhibitory molecule which mediates the sequential steps involved in angiogenesis. Lower level or impaired function of endostatin is associated with a higher risk of developing malignant solid tumors and with a worse prognosis of the disease. The *endostatin* N104 polymorphism might be associated with an impaired ability to inhibit angiogenesis. We analyzed the tissues from 98 Caucasian prostate cancer patients for the presence of D104N polymorphism. The frequencies of homozygous 4349G/G(104D/D), and heterozygous 4349G/A(104D/N) were 83.67%(82/98) and 16.33%(16/98), respectively; no individuals were homozygous 4349A/A(104N/N). With the Fisher's exact test we found the genotype of D104N was not significantly related to age, tumor grade, PSA and clinical stage ($P > 0.05$). There was no difference in relapse free survival(RFS) or overall survival(OS) between patients with 104D/N and those with 104D/D ($P = 0.8283$, 0.3713 respectively). We concluded that *endostatin* polymorphism was not associated with the aggressiveness of prostate cancer in Caucasian patients.

Keywords: Prostate cancer, endostatin, polymorphism

1. Introduction

The latest estimates of global cancer incidence show that prostate cancer has become the third most common cancer in men, with half a million new cases each year, almost 10% of all cancers in male [1–4]. Prostate cancer is usually regarded as a slow-growing tumor, and only 2.9% actually will die of the disease [5]. In order to better design therapeutic strategies for individual cases and identifying patients who would benefit from more vigilant surveillance, it would be of interest to study markers which can predict the outcome of disease. Angiogenesis, or the formation of new blood vessels from preexisting endothelium, is a fundamental step in tumor progression and metastasis [6,7]. Endostatin, a Mr 20,000 cleavage product of the COOH-terminal domain of collagen XVIII(NC1), has been considered as an important inhibitory molecules that can medicate the sequential steps involved in angiogenesis. Higher serum levels of endostatin induced experimentally in mice and rats seem to cause regression of various types of solid tumors, including prostate cancer [8–10]. In addition, Down's syndrome patients, who have a higher serum levels of endostatin because of their three copies of the

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Table 1
Associations of endostatin polymorphisms with other clinical and pathological parameters

Factors	4349 polymorphism		*P* value
	4349G/G(104D/D) n(%)	4349G/A(104D/N) n(%)	
Age (y)			
⩽60	30(88.24)	4(11.76)	0.6886
60–70	42(80.77)	10(19.23)	
>70	10(83.33)	2(16.67)	
PSA			
⩽4	12(70.59)	5(29.41)	0.2791
4–10	45(86.54)	7(13.46)	
>10	25(86.21)	4(13.79)	
Gleason grade			
⩽6	54(87.10)	8(12.90)	0.2640
> 6	28(77.78)	8(22.22)	
Clinical stage			
I	7(77.78)	2(22.22)	0.7479
II	57(85.07)	10(14.93)	
III	18(81.82)	4(18.18)	

COL18A1 gene, have a decreased incidence of solid tumors, including prostate cancer [11]. Thus, lower levels or an impaired function of endostatin might be associated with a higher risk of developing malignant solid tumor or with a worse prognosis of the disease. Visakorpi et al. conducted a systematic analysis of the COL18A1 gene and found a missense mutation of D104N located in the COOH-terminal globular domain NC1 of collagen XVIII, the encoding region of *endostatin* [12]. Iughetti et al. found that heterozygous N104 individuals have a higher chance of developing prostate cancer compared with homozygous D104 subjects and proposed the presence of N104 impairs the function of endostatin [13]. In the present study we hypothesize that N104 may impair the function of endostatin and in turn affect the aggressiveness of prostate cancer. To testify our hypothesis we assessed the *endostatin* polymorphism present in 98 Caucasian patients with prostate cancer to determine whether D104N is correlated with well known prognostic factors as well as relapse free survival(RFS) and overall survival(OS).

2. Materials and methods

2.1. Patient population and clinical data

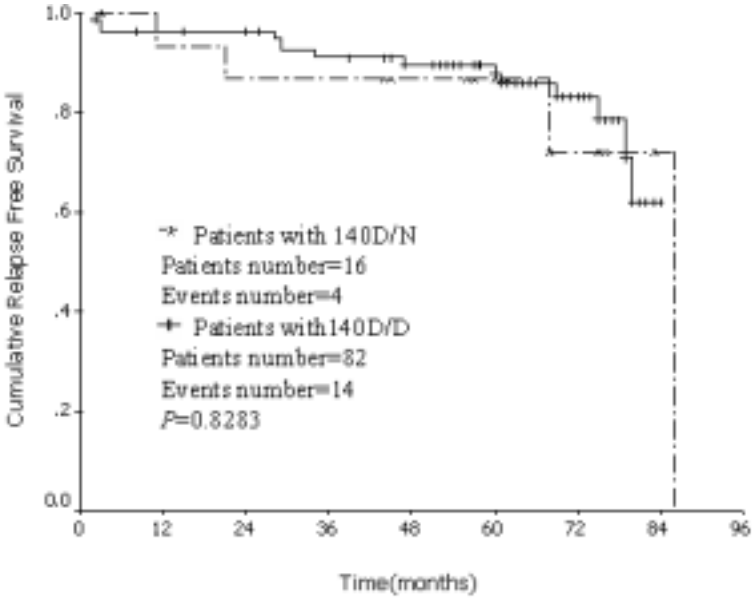
The study population consisted of 98 Caucasian patients with prostate cancer who underwent prostatectomy at Vanderbilt Hospital in 1997. The study was approved by Institutional Review Board (IRB# 030986) at Vanderbilt University School of Medicine. All patients had clinically localized prostate cancer and were treated with radical prostatectomy as a primary treatment. All patients had adenocarcinoma confirmed histologically. The patients were followed at Vanderbilt Hospital or at local hospitals with a mean follow-up of 62 months. Patients' age ranged 40 to 81 with a median age of 63 years. All pathological information was reviewed by one pathologist and the tumor differentiation was evaluated using Gleason's score criteria. Clinical stage was classified according to the AJCC TNM staging system [14]. The Clinical and histological characteristics of the patients are summarized in Table 1.

2.2. Tissue preparation and DNA extraction

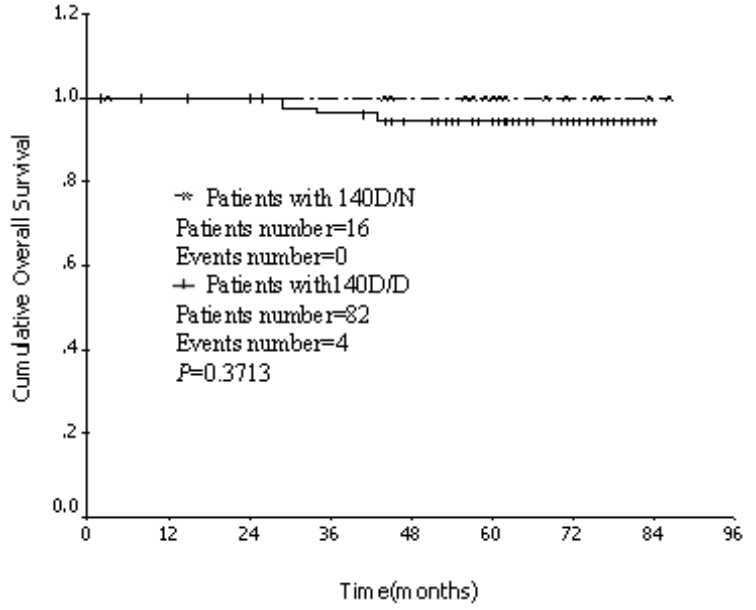
Using a standard microtome with disposable blades, 5 um thickness sections of a representative areas of normal prostatic glands were cut from the paraffin embedded blocks and stained with Hematoxylin and eosin (H&E) and then examined under a microscope to verify the absence of prostate cancer. 5 um thickness section (about 1 ug) from each patient was used for DNA extraction. The sections were deparaffinized with xylene at room temperature for 30 minutes twice. Then the deparaffinized tissue was washed with 100% ethanol twice. After the ethanol had evaporated completely, the tissue was completely lysed with proteinase K. Then QIAamp DNA Mini Kit (QIAGEN Inc, Valencia, CA) was used to extract extraction and purify DNA from the tissues according to the tissue protocol of the kit.

3. Polymorphism genotyping

The allelic discrimination of the *endostatin* gene 4349G/A polymorphism was assessed with the ABI



(A)



(B)

Fig. 1. Relapse free survival and overall survival curves. (A) RFS curves for patients with 4349G/G(140D/D) genotype and those with 4349G/A(140D/N); (B) OS curves for patients with 4349G/G(140D/D) genotype and those with 4349G/G (140D/N).

PRISM 7900 HT Sequence Detection System (Applied Biosystems). PCR was performed with a total volume of 5 ul, which contained approximately 2.5 ng DNA, 1 × Taqman Universal PCR Master Mix, each primer at a concentration of 900 nM, and each probe at a concentration of 200 nM. Primers for *endostatin* were from Applied Biosystems (Assay ID: C_11872896_10). Taqman probes were: A allele specific: 5′-VIC-GCC CGG GGC ACG CAT CTT CTC CTT TAA CGG CAA GGA CGT CCT GAG GCA CCC -NFQ-3′.G-allele specific:

5′-FAM- GCC CGG GGC ACG CAT CTT CTC CTT TGA CGG CAA GGA CGT CCT GAG GCA CCC -NFQ-3′. The thermal cycling conditions were as follows: 95°C for 10 min to activate the AmpliTaq Gold enzyme, followed by 40 cycles of 15 seconds at 95 °C and 60 seconds at 60°C. The fluorescence levels were measured with an ABI PRISM 7900 HT Sequence Detector (Applied Biosystems, Foster City, CA), resulting in clear identification of three genotypes of each polymorphism.

The laboratory staff was blind to the identity of the subjects. Quality control (QC) samples were included in the genotyping assays. Each 384 well plate contained four water, eight CEPH 1347-02 DNA, eight blinded QC samples, and eight unblinded QC samples. The blinded and unblinded QC samples were taken from the second tube of study samples included in this study.

4. Statistical analysis

Relapses free survival (RFS) was defined as the time between the date of the primary surgery to the date of relapse or the date of last follow-up. Overall survival (OS) was defined as time between the date of the primary surgery to the date of death or the date of last follow-up. The Kaplan-Meier method was used to compute 5-year survival rates and log-rank test was applied to test the difference in survival across different genotype. The associations between the *endostatin* polymorphism and other clinical and pathological characters was analyzed by Fisher's exact test. P-values $<$ 0.05 were considered statistically significant. All statistical analyses were two sided. We conducted the statistical analysis with SAS 9.1 *SAS* Institute Inc., Cary, NC.

5. Results

5.1. The correlation of 104D/N polymorphism with other clinical and pathological parameters

With Taqman SNP genotyping assay, the concordance of the blinded samples was 100%. Genotypes for 4349G/A polymorphism were successfully determined in 98 samples. The frequencies of homozygous 4349G(104D/D), and heterozygous 4349G/A(104D/N) were 83.67%(82/98) and 16.33%(16/98), respectively. With Fisher's exact test, *Endostatin* polymorphism was found to have no significant relationship with age ($P =$ 0.6886), PSA ($P = 0.2791$), Gleason's grade ($P =$ 0.2640) and clinical stage ($P = 0.7479$) (Table 1).

5.2. Survival analysis

The Kaplan-Meier method was used to compute survival rates and the log-rank test was applied to test the difference in survival across different genotypes. 5-year relapse free survival rates and overall survival rates for this group of patients were 87.71% and 95.64% respectively. There was no significant difference between subjects with D104N and those with D104D in RFS and OS (Log-rank test, $P = 0.8283$ and 0.3713 respectively) (Fig. 1A,B).

6. Discussion

In the current study, we analyzed the 4349G/A (D104N) polymorphism in 98 Caucasian patients and did not find any association between the polymorphism and other clinical and pathological variables, and we did not find any significant association between 4349G/A(D104N) polymorphism and patients' survival (RFS and OS) either.

Higher serum levels of endostatin induced experimentally in mice and rats seem to cause regression of various types of solid tumors, including prostate cancer [8–10]. Clinical study also showed that individuals with higher levels of endostatin might be less prone to develop solid tumors [15]. The missense mutation of D104N located in the COOH-terminal globular domain NC1 of collagen XVIII was found to be in the encoding region of *endostatin* by Visakorpi et al. in 1999 [12]. It has been recently shown that gene polymorphisms affect cancer cell proliferation, differentiation as well as tumor invasion/metastasis [16]. The possible association of D104N with cancer risk and prognosis remains poorly investigated. Iughetti et al reported that individuals heterozygous for N104 have a 2.5 times greater chance of developing prostate cancer as compared with homozygous D104 subjects (OR: 2.4, 95%CI:1.4–4.16); the author proposed that *endostatin* N104 has a decreased affinity for binding to other molecules and an impaired ability to inhibit angiogenesis [13]. In a case-control study containing 58 multiple myeloma patients, Ortega et al reported that the genotype frequencies were in Hardy-Weinberg equilibrium and that the D104N polymorphism was an unimportant determinant in multiple myeloma patients [17]. Liu et al. reported a case control study including 274 patients with leukemia and 178 normal controls. In Liu's study there were no patients homozygous for 4349A which was similar to our study; they found no association with

endostatin polymorphism and the risk of leukemia [17, 18]. Most of the previous studies showed there were no association between the D104N polymorphism and malignancies. Only Iughetti et al. reported a higher risk ratio of developing prostate cancer for heterozygous N104 individuals, but even in this study they did not find any association between the presence of the mutation and Gleason score or age at diagnosis. We not only further confirm D104N polymorphism had no relationship with Gleason score or age at diagnosis, but also demonstrate that the D104N polymorphism has no relationship with PSA level, clinical stage as well as RFS and OS of patients.

The discrepancies between our study and previous studies might due to several reasons. The translation of results from different studies might be limited due to the limited sample size and different patient selection. In our study we selected stage I or II Caucasian patients. Another reason is that we analyze the relationship between the *endostatin* polymorphism and prostate cancer from the aspect of aggressiveness of disease but not cancer risk and this will also cause the discrepancies between our study and previous ones. Last but not the least reason, most of the previous studies as well as ours were retrospective and the conclusions must be drawn cautiously. So far, to the authors' knowledge there is no data from prospective randomized study available.

In conclusion, our study indicated that the *endostatin* polymorphism 4349G/A(D104N) is not associated with aggressiveness of disease in Caucasian patients with prostate cancer.

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