A high risk of osteosarcoma in individuals who are homozygous for the p.D104N in endostatin

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The D104N polymorphism (p.D104N) in endostatin has been previously identified in many types of cancer, and this polymorphism is believed to be a phenotypic modulator in some tumors. However, it is unknown whether endostatin p.D104N affects the risk and progression of osteosarcoma (OS). Here, we analyzed the p.D104N endostatin variant in 236 patients with OS and 418 healthy individuals. Similar frequencies of wild type and heterozygous p.104DN endostatin were observed in controls and OS patients. Interestingly, the frequency of the homozygous p.D104N (p.104NN) genotype was higher in OS patients group compared to control group, suggesting that individuals with p.104NN endostatin have a significantly increased risk for OS. In addition, OS patients with p.104NN endostatin had a shorter survival time and a higher rate of metastasis than OS patients with wild type endostatin. Animal experiments revealed that overexpression of p.104NN endostatin did not significantly inhibit OS lung metastasis. Interestingly, administration of endostatin dramatically inhibited OS lung metastasis in the p.104NN endostatin xenograft model. Together, these results suggest that p.104NN of endostatin is associated with the risk of OS and demonstrates predictive significance for clinical outcome in OS patients. In addition, endostatin therapy may be necessary for OS patients harboring p.104NN endostatin.

Osteosarcoma (OS) is the most common primary malignant tumor in children1, and 30% of children diagnosed with OS will not survive for more than 5 years2-3. Treatment of this disease often fails due to the development of metastasis4. However, the cellular mechanisms that underlie the development and metastasis of OS remain unclear.

Angiogenesis is a discrete event in carcinogenesis that is related to the aggressive potential of a tumor5,6. Accumulating evidence suggests that tumor growth is associated with increased angiogenesis and that the formation of new blood vessels is a fundamental step in tumor development and expansion7. In addition, studies have suggested that increased angiogenic properties within a tumor are associated with poorer clinical outcomes8. As is the case for numerous types of cancer, OS is dependent on angiogenesis9. Therefore, anti-angiogenic therapy has become a vital part of the arsenal against OS10,11.

Endostatin is a 183-amino-acid proteolytic fragment that is produced by the cleavage of the C-terminal non-collagenous domain (NC1) of human type XVIII collagen; endostatin is an efficient anti-angiogenic molecule12. As an angiogenic inhibitor, endostatin prevents tumor growth and expansion by controlling...
In the 236 OS patients (272.4 ng/ml) was higher than that in the 100 healthy individuals (95.1 ng/ml). A single nucleotide polymorphism, c.4309G > A (p.D104N), has been identified in the endostatin domain of COL18A1; this polymorphism affects a site that is conserved in humans and mice. A polymorphism can influence gene function and recent studies show that some polymorphism is strongly associated with the clinical significance in cancers. So, the frequencies of endostatin p.D104N variant has been previously evaluated in healthy individuals and various diseases including cancers. Iughetti et al. reported that the presence of the p.D104N variant results in a 2.5-fold increased risk of prostate cancer. In addition, studies in breast cancer showed that endostatin p.D104N is associated with invasive breast cancer, and patients carrying this polymorphism exhibited shorter progression-free survival and overall survival compared to those with wild type endostatin. However, it is not known whether this polymorphism in endostatin affects the risk and progression of OS.

Therefore, we aimed to determine the frequency of the wild type, heterozygous p.D104N (p.104DN), and homozygous p.D104N (p.104NN) endostatin genotypes in patients with OS and to determine the influence of this polymorphism on OS risk, biological features and clinical outcomes.

### Results

#### Distribution of the p.D104N endostatin variant in OS patients.

First, we analyzed the distribution of the p.D104N endostatin variants in the healthy control and OS patient groups (Table 1). Of the 236 OS patients, 205 were normal homozygous (wild type) and 23 were heterozygous for the p.D104N. Of the 418 healthy individuals, 376 harbored the wild genotype of endostatin, 39 had the p.104DN genotype and 3 had the p.104NN genotype. We observed no significant differences in the frequencies of the wild type and p.104DN genotypes between the OS patient group and the healthy control group. However, we observed a higher frequency of individuals who were homozygous for p.D104N in the OS patient group (3.4%) than in the control group (0.7%) (Table 1).

| Genotypes     | Controls number (%) | Patients Number (%) |
|---------------|---------------------|---------------------|
| p.104NN       | 3 (0.7)             | 8 (3.4)             |
| p.104DN       | 39 (9.3)            | 23 (9.7)            |
| Wild          | 376 (90.0)          | 205 (86.9)          |

Table 1. Endostatin genotype distribution among osteosarcoma patients and controls. Wild, no polymorphism; p.104DN, heterozygous polymorphism; p.104NN, homozygous polymorphism.

The formation of new blood vessels. In vitro and in vivo studies have shown that endostatin treatment can increase apoptosis and decrease microvessel density and metastasis in many tumors, including OS. A single nucleotide polymorphism, c.4309G > A (p.D104N), has been identified in the endostatin domain of COL18A1; this polymorphism affects a site that is conserved in humans and mice. A polymorphism can influence gene function and recent studies show that some polymorphism is strongly associated with the clinical significance in cancers. So, the frequencies of endostatin p.D104N variant has been previously evaluated in healthy individuals and various diseases including cancers. Iughetti et al. reported that the presence of the p.D104N variant results in a 2.5-fold increased risk of prostate cancer. In addition, studies in breast cancer showed that endostatin p.D104N is associated with invasive breast cancer, and patients carrying this polymorphism exhibited shorter progression-free survival and overall survival compared to those with wild type endostatin. However, it is not known whether this polymorphism in endostatin affects the risk and progression of OS.

Therefore, we aimed to determine the frequency of the wild type, heterozygous p.D104N (p.104DN), and homozygous p.D104N (p.104NN) endostatin genotypes in patients with OS and to determine the influence of this polymorphism on OS risk, biological features and clinical outcomes.
observe any differences in endostatin expression in the tumor tissues from the OS patients with different p.D104N genotypes (Fig. 3d).

The p.104NN genotype does not affect the anti-angiogenesis function of endostatin in OS. Endostatin inhibits tumor metastasis partly by inhibiting angiogenesis\(^{26}\), and p.D104N potentially impairs endostatin function\(^{21}\). Thus, we examined the effects of the p.104NN genotype on the anti-angiogenic activity of endostatin. First, we measured the microvessel density in tissue samples from OS patients with different endostatin p.D104N variants. Angiogenesis was slightly increased in OS patients with the p.104NN genotype, but the differences among these OS patients were not significant (Fig. 4a). In agreement with the clinical data, the animal experimental results showed that p.104NN did not significantly affect the anti-angiogenic activity of endostatin (Fig. 4b). Together, these data indicated that p.104NN does not affect the anti-angiogenesis function of endostatin.

| Sex     | Patients number | Wild+p.104DN(%) | p.104NN(%) | p-value |
|---------|----------------|----------------|------------|---------|
| Male    | 139            | 134(96.4)      | 5(3.6)     | 1.00    |
| Female  | 97             | 94(96.9)       | 3(3.1)     |         |
| Age     | 1.00           |                |            |         |
| 20≤     | 162            | 157(96.9)      | 5(3.1)     |         |
| >20     | 74             | 71(95.9)       | 3(4.1)     |         |
| Histologic subtype | |                |            | 0.65    |
| Osteoblastic | 146            | 142(97.3)      | 4(2.7)     |         |
| Chondroblastic | 35             | 33(94.3)       | 2(5.7)     |         |
| Fibroblastic  | 21             | 20(95.2)       | 1(4.8)     |         |
| Other   | 34             | 33(97.1)       | 1(2.9)     |         |
| Grade   | 1.00           |                |            |         |
| III     | 61             | 59(96.7)       | 2(3.3)     |         |
| IV      | 175            | 169(96.6)      | 6(3.4)     |         |
| Enneking stage | |                |            | 0.72    |
| 2A      | 59             | 56(94.9)       | 3(5.1)     |         |
| 2B      | 177            | 172(97.2)      | 5(2.8)     |         |

Table 2. Endostatin genotype distributions in osteosarcoma patients stratified by clinical and laboratory variables. Wild, no polymorphism; p.104DN, heterozygous polymorphism; p.104NN, homozygous polymorphism.

![Figure 1. Homozygous D104N polymorphisms (p.104NN) in endostatin are associated with reduced survival and a high metastatic rate.](image)

(a) The survival rate was determined using the Kaplan-Meier method. The survival rate was significantly lower in osteosarcoma (OS) patients with p.104NN endostatin compared to OS patients who were heterozygous for the D104N polymorphism (p.104DN) or who harbored wild type endostatin. (b) The OS metastasis rate was higher in OS patients with p.104NN endostatin compared to OS patients with p.104DN or wild type endostatin.
Discussion

The D104N polymorphism in endostatin has been previously reported in many types of cancer, and this polymorphism is believed to be a phenotypic modulator in some benign and malignant tumors. However, these data are controversial, and different results have been reported in different cancer types. For example, studies have shown that the heterozygous p.D104N of endostatin is associated with an increased risk of prostate cancer and invasive breast cancer and with worse clinical outcomes for...
gastric cancer, whereas no associations were observed between the heterozygous p.D104N genotype and the risk of multiple myeloma and lung cancer. These discrepant results may be due to the heterogeneity of different cancers. This is the first study to report the frequency of the p.D104N polymorphism in endostatin.

Figure 3. The D104N polymorphism (p.D104N) does not affect endostatin expression. (a) Serum endostatin levels were significantly higher in osteosarcoma (OS) patients (n = 236) compared to healthy individuals (n = 100). (b) The p.D104N does not affect serum endostatin levels in healthy individuals. (c) The p.D104N does not affect serum endostatin levels in OS patients. (d) The p.D104N does not affect endostatin expression in OS tissue. Immunohistochemistry assays were performed on primary tumor tissues from OS patients. Dark brown color indicates endostatin expression. Each bar represents the mean ± SD. Wild, wild type endostatin; p.104DN, heterozygous D104N polymorphism; p.104NN, homozygous D104N polymorphism.

Figure 4. The D104N polymorphism in endostatin does not significantly affect angiogenesis in patients with osteosarcoma. (a) Microvessels were detected using CD31 immunofluorescence in primary tumor samples from OS patients. (b) Microvessels were detected in primary tumors from the LM8 xenograft model. Each bar represents the mean ± SD. Wild, wild type endostatin; p.104DN, heterozygous D104N polymorphism; p.104NN, homozygous D104N polymorphism.
in endostatin in OS patients and to ascertain whether p.D104N alters the risk and clinical manifestations of OS.

In the present study, we observed no differences in the frequencies of the wild type and p.104DN genotypes of endostatin between healthy control individuals and OS patients. Interestingly, we found that individuals with p.104NN had a significantly greater risk of disease occurrence. These data suggest that p.104NN endostatin may be associated with OS susceptibility. In addition, our data revealed that patients with p.104NN endostatin have a shorter survival time and a higher rate of metastasis, suggesting that the p.104NN genotype of endostatin is associated with poor clinical outcome. Our animal experimental data demonstrated that overexpressing p.104NN endostatin does not significantly inhibit OS lung metastasis. Together, these data suggest that p.104NN endostatin may be a useful candidate marker for predicting OS disease progression.

Interestingly, the animal experiments showed that endostatin treatment can significantly inhibit OS lung metastasis in the p.104NN endostatin OS xenograft model, suggesting that endostatin therapy may be useful for preventing OS lung metastasis in OS patients with p.104NN endostatin. However, we did not observe a significant association between the different p.D104N endostatin variants and tumor aggressiveness in the OS patients. This result may be due to the inclusion of relatively few individuals with the p.104NN variant in this study, suggesting future study is needed in the larger OS patient population. A similar result has been observed for other tumor types. Loureno et al. first reported that the p.104NN endostatin genotype is present in patients with sporadic breast cancer (SBC) but absent in control individuals, suggesting that the p.104NN genotype of endostatin is associated with SBC susceptibility. They also observed no association between the p.104NN endostatin genotype and tumor aggressiveness, which is similar to the results reported here.

According to previous reports, polymorphisms can alter gene expression and function. So, we hypothesized that the decreased anti-tumor effect of p.104NN endostatin may be due to downregulated expression or loss of function of endostatin. However, our data showed that p.104NN does not affect endostatin expression. Our results are supported by previously published studies. These data suggested that the effect of p.104NN on the anti-tumor activity of endostatin was not due to the inhibition of endostatin expression. Therefore, we examined the effects of p.104NN on the anti-angiogenic activity of endostatin. Endostatin is an anti-angiogenic factor, and studies have shown that the antitumor effects of endostatin are mediated partly by inhibiting angiogenesis. Unfortunately, angiogenesis was slightly increased in patients with p.104NN endostatin compared to patients with wild type endostatin. Similar results were observed in the animal experiment, suggesting that the diminished anti-tumor effects of p.104NN endostatin are not due to modulation of anti-angiogenic activity. Thus, the mechanisms by which p.104NN weakens the anti-tumor effects of endostatin are unclear, and future studies are needed.

In summary, our study is the first to report preliminary evidence that the p.104NN genotype of endostatin is associated with OS susceptibility and poor clinical outcome and that it has predictive significance for the clinical outcome of OS patients. In addition, endostatin therapy may be necessary for OS patients with p.104NN endostatin.

Methods

Materials. EDTA-containing vacutainer tubes were obtained from BD vacutainer (Franklin Lake, NJ, USA). Genomic DNA isolation kits were purchased from Qiagen (Germantown, MD, USA). Endostatin human ELISA kits and primary antibodies against CD31 and endostatin were obtained from Abcam (Cambridge, MA, USA). Permount, Hematoxylin and secondary antibodies conjugated to HRP or FITC green were obtained from Sigma (St. Louis, MO, USA). DAB peroxidase substrate kits and DAPI-containing mounting media for immunofluorescence were purchased from Vector Labs (Burlingame, CA, USA). Endostatin was obtained from Shandong Xiangsheng Maideljin Biological Pharmaceutical Co.

Human specimens. All the experimental methods were carried out in accordance with the approved guidelines. Blood and tumor samples were obtained from 236 OS patients primary tumors during diagnostic surgical biopsies (no metastasis). Blood samples were also obtained from 418 healthy individuals. The characteristics of the OS patients and the controls are summarized in Table 3. This research was approved by the Research Ethics Board of the General Hospital of the People’s Liberation Army. After describing the research study and the related procedures, written informed consent was obtained from the adult patients or from legally authorized representatives if the patients were minors.

Genomic DNA amplification and genotyping. Genomic DNA was isolated from the blood samples (healthy individuals) and the diagnostic biopsy specimens (OS patients) using a Qiagen genomic DNA isolation kit according to the manufacturer’s instructions. Genotyping was performed as described by Balasubramanian et al. using the same primers.

Immunostaining assay. The tumor tissues were fixed in 10% neutral buffer formalin, embedded in paraffin, and sectioned at a thickness of 4μm. The tissue sections were deparaffinized in xylene and then rehydrated via an alcohol gradient. Antigen retrieval was then performed using 10mM citrate buffer (pH 6.0). For immunofluorescence (IF), the tissue sections were directly incubated in a solution of 3% bovine serum albumin (BSA) in PBS for 1 h at room temperature (RT). For immunohistochemistry
(IHC), tissue sections were incubated in 3% hydrogen peroxide in methanol for 10 min and then washed with PBS. Then, the tissue sections were incubated in a 3% BSA solution. The slides were then incubated with a primary antibody overnight at 4 °C. The following day, the tissue sections were washed and incubated with the secondary HRP-conjugated (for IHC) or FITC-conjugated (for IF, in the dark) antibodies at RT. After a 1-h incubation, the slides were washed with PBS in the dark. For IF, a cover slip was affixed using mounting medium with DAPI. For IHC, the substrate color was developed using a DAB peroxidase substrate kit, and the sections were counterstained with hematoxylin. Next, cover slips were mounted using Permount. The number of CD31-positive vessels was counted in four randomly selected 1 mm² areas in a high power field (HPF), and the average number was calculated. To quantify the total surface area of the vasculature, the total perimeter of the vessels was measured in five randomly selected 0.25 mm² areas using an image analyzer. Endostatin-positive staining was determined by counting 5 randomly chosen fields per section, and the percentage of DAB-positive cells per 100 cells was determined at ×400.

**ELISA assays.** Blood samples were collected in EDTA-containing tubes, and the serum samples were separated and stored at −70 °C for future use. Serum endostatin levels were measured using a commercially available ELISA according to the manufacturer's instructions. All of the measurements were performed in duplicate to ensure the accuracy of the collected data.

**Animal study.** An animal study was performed as described by Kaya et al. Briefly, female BALB/c nu/nu mice (8 mice/group) were inoculated subcutaneously with LM8 cells that had been transfected with the indicated endostatin variant (1 × 10⁶ cells/mouse). One week after inoculation, blood samples were collected from the tail vein and analyzed for endostatin. At the indicated times after inoculation, the mice were anesthetized, and the primary tumors were removed surgically. The lungs were removed 2 weeks after surgery, and the macroscopic pulmonary metastatic foci were counted.

For the endostatin therapy experiment, the mice were inoculated subcutaneously with p.104NN endostatin-overexpressing LM8 cells (1 × 10⁶ cells/mouse). Two weeks after inoculation, the mice were anesthetized, and the primary tumors were removed surgically. After the primary tumor was removed, endostatin was administered to the treatment group by intraperitoneal injection (5 mg/kg, every 2 days). The control group received physiological saline. The lungs were removed 2 weeks after endostatin treatment, and the macroscopic pulmonary metastatic foci were counted. The experimental animal protocol was approved by the institutional review board of the General Hospital of the People's Liberation Army and was performed in accordance with accepted guidelines for animal research.

**Statistical analyses.** Hardy-Weinberg equilibrium was evaluated using the X² statistic for the goodness-to-fit (using one degree of freedom). The statistical significance of the differences between groups was calculated using the X² test or Fisher's exact test. All the analyses were performed using the SAS statistical package for Windows, version 8.1 (SAS Institute Incorporation, USA).
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Author Contributions
C.X.X., M.X. and D.W.L. contributed to the conception and design of the study; W.Z.B., M.X., D.W.L., Y.W. and S.L. collected and provided the human samples; W.Z.B., H.J., L.M.X., B.S. and Y.W. performed laboratory experiments; Z.G.S., C.I., Q.L. and D.W. analyzed clinical data; C.X.X., H.J. and M.X. discussed the results and wrote the manuscript.

Additional Information
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