Osteopontin Promoter Polymorphisms at Locus -443 Significantly Affect the Metastasis and Prognosis of Human Hepatocellular Carcinoma

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Osteopontin (OPN) plays a crucial role in hepatocellular carcinoma (HCC) metastasis. However, little is known about the impact of OPN polymorphisms on cancer progression. In this study, we first identified the single nucleotide polymorphisms (SNPs) in the OPN promoter region by direct sequencing in 30 HCCs, and then evaluated the prognostic values of the selected ones in two large cohorts of 826 HCC patients. The identified SNPs were functionally analyzed using in vitro and in vivo assays and their correlations with OPN levels were also evaluated. Only SNP at locus -443 and their related haplotypes (Ht2: -1748A/-616G/-443T/-155* [*indicates base deletion]; Ht3: -1748A/-616G/-443C/-155*) were significantly associated with overall survival (OS) and time to recurrence (TTR). The patients with the -443TT/TC genotype or Ht2 had a shorter OS and TTR compared with those with -443CC genotype or Ht3. This was further confirmed in the validation cohort. Moreover, this correlation remained significant in patients with small HCCs (<5 cm). Multivariate analyses indicated that the prognostic performance of the -443 genotypes (OS, \( P < 0.031 \); TTR, \( P = 0.005 \)) and their related haplotypes (OS, \( P = 0.002 \); TTR, \( P = 0.001 \)) was independent of other clinicopathological factors. The Ht2 and -443TT genotype could significantly increase the promoter transcriptional activity and expression level of OPN compared with the Ht3 or -443CC genotype, and lead to an obvious increase in both in vitro invasion and in vivo tumor growth and lung metastasis of HCC cells (\( P < 0.05 \)).

**Conclusion:** The genetic variation at locus -443 of the OPN promoter plays important roles in the regulation of OPN expression and cancer progression of HCCs, which is a novel determinant and target for HCC metastasis and prognosis.

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**Abbreviations:** AFP, alpha-fetoprotein; DMEM, Dulbecco’s modified Eagle’s medium; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HCC, hepatocellular carcinoma; LD, linkage disequilibrium; OPN, osteopontin; OS, overall survival; SNP, single nucleotide polymorphism; TTR, time to recurrence.

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**During the past decades, although much progress has been achieved in the clinical management of hepatocellular carcinoma (HCC), its prognosis remains dismal.**1,2 Thus, to develop effective individualized treatments based on molecular classification is pivotal to improve the prognosis of HCC patients.

Osteopontin (OPN) is a secreted noncollagenous, sialic-acid-rich, chemokine-like extracellular matrix (ECM) protein. OPN binds to \( \alpha \beta \) integrins and receptors of the CD44 family to promote cell adhesion, chemotaxis, ECM degradation, angiogenesis, prevention of apoptosis, and indolent tumor growth.3 Moreover, it plays a crucial role in determining the oncogenic potential of various cancers, contributing to tumor invasion and metastasis.4-6 In HCC increased OPN levels are associated with metastasis, poor prognosis, and early tumor recurrence.7-9
The human OPN gene is localized on chromosome 4q13. More than 10 single nucleotide polymorphisms (SNPs) have been identified in the promoter. These polymorphisms may affect the transcriptional activity of OPN, and some of them are thought to be genetic risk factors for disease susceptibility. This suggests that OPN, and some of them are thought to be genetic risk factors for disease susceptibility.

Patients and Methods

Patients and Specimens. A total of 856 patients who underwent curative resection for HCC at the Liver Cancer Institute and Zhongshan Hospital, Fudan University (Shanghai, China) from January 2002 to December 2006 were enrolled in the present study. Thirty of them were used to screen the polymorphisms in the promoter region of OPN. The remaining 826 patients were divided into two cohorts: 1) the training cohort containing 317 patients was used to evaluate the prognostic significance of the selected SNPs for human HCCs; 2) the independent validation cohort of 509 patients was used to further validate their prognostic values. No patients received any preoperative cancer treatment and were followed up until May 2011, with a median follow-up of 53.0 months (range 2-110 months). During the follow-up, patients were monitored every 2-3 months after the operation. Computed tomography (CT) scanning or magnetic resonance imaging (MRI) was performed when tumor recurrence was suspected. A further treatment was given if tumor relapse was diagnosed, as described. The detailed clinicopathological characteristics of the study participants are presented in Supporting Table S1. There was no significant difference in the clinicopathologic features between the two cohorts.

Snap-frozen or paraffin-embedded tissue specimens were obtained from these patients after obtaining written informed consent. All tumors were proven to be HCC by two pathologists. This study was approved by the Research Ethics Committee of Zhongshan Hospital, Fudan University.

DNA Extraction, SNP Genotyping, and Haplotypes Reconstruction. Genomic DNA was isolated from tissue samples with QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Polymorphisms were screened in 30 HCC samples by direct sequencing ~2.4 kb upstream from the transcription start site of OPN. Genotyping by pyrosequencing was performed with the PSQ 96 system (Biotage, Uppsala, Sweden) in two independent cohorts of patients. Then the haplotypes were analyzed with PHASE software v. 2.1. The linkage disequilibrium (LD) between OPN polymorphisms was determined with the Haplovie program v. 3.2. Haplotype blocks were defined according to the “spine of LD” setting in Haplovie software, on the basis of each end marker of a block having a D’ value greater than 0.8.

Construction and Promoter-Activity Assays of Luciferase Reporter Vectors With Specific Haplotypes of OPN. Two promoter-reporter plasmids were constructed from genomic DNA with Ht2 (-443T allele) or Ht3 (-443C allele) native haplotypes. The OPN promoter-reporter constructs were transfected into HCC cells together with the internal control pRL-CMV. The luciferase activities were measured with the Dual-Glo luciferase kit (Promega). The HCC cells transfected with pGL3-Basic plasmid were used as mock control. Details are in the Supporting Materials and Methods.

Immunohistochemical Staining for OPN. Formalin-fixed and paraffin-embedded tissues were used for immunohistochemical staining, as described in the Supporting Materials and Methods.

Detection of Protein Levels by Western Blot. The protein expression levels of OPN and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were evaluated by western blot, as described in the Supporting Materials and Methods.

In Vitro Invasion Matrigel Assays. The invasive ability of HCC cells was determined using Matrigel (BD Pharmingen)-coated 24-well transwell chambers. Details are described in the Supporting Materials and Methods.

In Vivo Assays for Tumor Growth and Metastasis. HepG2 cells (5 x 10⁶) transfected with promoter-reporter constructs (OPN-Ht2, OPN-Ht3, control) were implanted subcutaneously into the flank of nude mice (BALB/c nu/nu, 4-6 weeks). Tumor growth was monitored with tumor volume, which was calculated as described. The mice were sacrificed 6 weeks later and the lungs removed. Consecutive sections were made for every tissue block of the lung and stained
with hematoxylin-eosin. The incidence and classification of lung metastases were calculated and evaluated independently by two pathologists. Based on the number of HCC cells in the maximal section of the metastatic lesion, the lung metastases were classified into four grades20,21: grade I, <20 tumor cells; grade II, 20-50 tumor cells; grade III, 50-100 tumor cells; and grade IV, >100 tumor cells. These procedures were approved by the Animal Care and Use Committee of Fudan University, China.

Statistical Analysis. Analysis was performed with SPSS 15.0 for Windows (Chicago, IL). Overall survival (OS) and time to recurrence (TTR) were compared with the Kaplan-Meier method and significance was determined by the log-rank test. The Cox regression model was applied to evaluate the effects of each clinical variable, OPN genotypes and haplotypes on TTR or OS. A receiver operating characteristic (ROC) curve was used to determine the prediction value of a parameter. Harrell’s concordance index (C-Index) was to measure the discriminatory ability of the Cox regression model. Values are expressed as the mean ± standard deviation. P < 0.05 was considered statistically significant.

Results

Polymorphisms and Haplotypes Identified in OPN Promoter. To explore the sequence variation in the OPN promoter, ~2,400 basepairs upstream from the transcription start site were amplified by polymerase chain reaction (PCR) from the genomic DNAs of 30 HCC samples and then sequenced. Four SNPs at loci -1748, -616, -443, and -155 of OPN promoter were identified as candidates for further evaluation (Supporting Table S2). The SNPs at loci -1748, -616, and -155 were in perfect linkage disequilibrium (D’ >0.8), but this linkage disequilibrium did not include the SNP at locus -443 (Supporting Fig. S1). A total of 14 haplotypes were identified by PHASE analysis, but only six occurred with a frequency greater than 1% (Supporting Table S2). Three major haplotypes (Ht1: -1748G/-616T/-443T/-155G; Ht2: -1748A/-616G/-443T/-155* [indicates base deletion]; Ht3: -1748A/-616G/-443C/-155*) accounted for 89% of the genetic variations. Moreover, it is very interesting that only one different nucleotide at locus -443 (T/C) was found between the two major haplotypes, Ht2 and Ht3. More frequent TT (57.4%) and TC (34.1%) genotypes were found at locus -443 compared with the CC genotype (8.5%) (Supporting Table S2). These findings were confirmed in the following validation cohort.

Association of OPN Promoter Polymorphisms With Prognosis of HCC Patients. The associations of polymorphisms found above in the OPN promoter with OS and TTR were investigated in 317 HCC patients of the training cohort. In the Kaplan-Meier analyses, SNP at locus -443 was significantly associated with OS and TTR. The patients with -443TT/TC genotype exhibited a decreased postoperative OS and a shorter TTR compared with those with the -443CC genotype (Fig. 1A). The 1-, 5-, and 9-year OS rates of the patients with -443TT/TC genotype were 81.3%, 51.2%, and 37.0%, respectively, which were significantly lower than those with the -443CC genotype (88.7%, 68.6%, and 68.6%, respectively; P = 0.027). The 1-, 5-, and 9-year cumulative recurrence rates of the -443TT/TC genotype group were 27.7%, 62.0%, and 69.8%, respectively, which were significantly higher than that of the -443CC genotype group (15.0%, 35.1%, and 35.1%, respectively; P = 0.005). In addition, the patients with homozygous Ht2 (Ht2/Ht2) had a significantly shorter OS (P = 0.002) and TTR (P = 0.004) than those with homozygous Ht3 (Ht3/Ht3) (Fig. 1B). No significant association was found between the OPN promoter polymorphisms at other positions and OS or TTR (Supporting Fig. S2).

To further evaluate the prognostic value of OPN promoter genotypes for HCC patients, univariate and multivariate analyses were performed with the clinicopathological characteristics and OPN promoter genotypes (Tables 1, 2). In the univariate analysis, serum alpha-fetoprotein (AFP) level, tumor size, tumor number, vascular invasion, TNM stage, BCLC stage, and tumor differentiation were revealed to significantly associate with OS and TTR of HCC patients. The -443 genotypes and their related haplotypes were also significantly associated with both OS and TTR. No significant prognostic significance was found in the other characteristics including sex, age, liver cirrhosis, HBsAg, tumor encapsulation, and the other SNPs of OPN promoter for OS or TTR (Table 1). In the multivariate analysis, the -443 genotypes and their related haplotypes were still revealed to be independent prognostic indicators for both OS (P = 0.031, P = 0.002, respectively) and TTR (P = 0.005, P = 0.001, respectively) (Table 2).

Moreover, the prognostic significances of -443 genotypes and their related haplotypes of OPN promoter still remained significant in the patients with small HCCs (<5 cm). Among the 191 patients with a small HCC in the training cohort, the 1-, 5-, and 9-year OS
Fig. 1. The association of -443 genotypes and their related haplotypes in OPN promoter with OS (left panel) and TTR (right panel) of HCC patients. (A) Kaplan-Meier analysis of the correlation between -443 genotypes and OS or TTR of HCC patients in the training cohort. (B) Kaplan-Meier analysis of the correlation between haplotypes and OS or TTR of HCC patients in the training cohort. (C) Kaplan-Meier analysis of the correlation between -443 genotypes and OS or TTR of HCC patients in the validation cohort. (D) Kaplan-Meier analysis of the correlation between haplotypes and OS or TTR of HCC patients in the validation cohort.
of the -443TT/TC genotype group (92.8%, 61.8%, and 43.2%, respectively) were significantly lower than those of the -443CC group (93.3%, 86.7% and 86.7%, respectively; \( P = 0.013 \)). The probabilities of recurrence at 1-, 5-, and 9-year of the TT/TC genotype group (15.5%, 53.6%, and 65.1%, respectively) were significantly higher than that of the CC genotype group (6.7%, 20.0%, and 20.0%, respectively; \( P = 0.006 \)) (Supporting Fig. S3A). Furthermore, the patients with homozygous Ht2 had a significantly shorter OS (\( P = 0.003 \)) and TTR (\( P = 0.002 \)) than those with homozygous Ht3 (Supporting Fig. S3B). Cox regression analyses indicated that the -443 genotypes and their related haplotypes were still independent prognostic indicators for both OS (\( P = 0.025, P = 0.003 \), respectively) and TTR (\( P = 0.014, P = 0.007 \), respectively) (Supporting Table S3).

In addition, we also found that the -443 genotypes and their related haplotypes could provide additional predictive abilities for clinical parameters and staging systems (e.g., BCLC and TNM) for both OS and TTR in terms of C-Index analysis (Fig. 2A). This indicates that the SNP at locus -443 and their related two haplotypes of OPN (Ht2 and Ht3) could significantly affect the prognosis of HCC patients.

### Validation of the Prognostic Values of OPN Promoter Polymorphisms at Locus -443 in an Independent Cohort of HCC Patients.

To further validate the prognostic significance of OPN promoter polymorphism at locus -443 and their related two haplotypes (Ht2 and Ht3), we analyzed their association with HCC prognosis in the independent validation cohort of 509 patients. Similar to the findings from the training cohort, the patients in the -443TT/TC genotype group had a significantly shorter OS and TTR than those of the -443CC genotype group (Fig. 1C). The 1-, 5-, and 9-year OS rates of the patients with -443TT/TC genotype (77.9%, 44.1%, and 31.7%, respectively) were significantly lower than that of the -443CC genotype group (89.4%, 60.3%, and 57.2%, respectively).
respectively; \( P = 0.008 \)). The tumor recurrence probabilities at 1-, 5-, and 9-year of the -443TT/TC genotype group (39.1%, 70.0%, and 83.5%, respectively) were significantly higher than that of the -443CC group (27.7%, 56.6%, and 65.8%, respectively; \( P = 0.013 \)). Similarly, the patients with homozygous Ht2 had a significantly shorter OS (\( P = 0.038 \)) and TTR (\( P = 0.006 \)) than those with homozygous Ht3 (Fig. 1D).

Likewise, in univariate and multivariate analysis the -443 genotypes and homozygous Ht2 were still independent prognostic indicators for both OS (\( P = 0.014, P = 0.028 \), respectively) and TTR (\( P = 0.023, P = 0.009 \), respectively) (Supporting Tables S5, 6). A C-Index analysis indicated that the combinations of them with the clinical characteristics and staging systems could enhance the predictive powers for OS and TTR (Fig. 2B).

The SNP at locus -443 was found to be associated with the aggressive histopathological characteristics of HCC, such as higher levels of AFP, larger tumor size, poorer tumor differentiation, vascular invasion, extrahepatic metastasis, and late BCLC stages. However, the association did not reach statistical significance except for tumor differentiation, tumor size, and BCLC stages (Supporting Table S7).

### Effects of OPN Promoter Polymorphisms at Locus -443 on the Promoter Activity and Expression Levels of OPN in HCCs.

To examine the biological effects of SNP at locus -443 of the OPN promoter, we established two stable cell lines transfected with promoter-reporter constructs containing the two major haplotypes, Ht2 (-443T allele) or Ht3 (-443C allele). The stable HCC cells transfected with empty pGL3-Basic vector were used as mock control. Assays of luciferase activities showed that the transcriptional activities of both OPN promoter-reporter constructs containing Ht2 or Ht3 were stronger than that of the control pGL3-Basic vector. Moreover, the luciferase activity of the reporter containing the Ht2 sequence was much higher than that containing the Ht3 sequence in HepG2 cells (\( P = 0.009 \)) (Fig. 3A). The same results were observed in the MHCC-97H cell lines (Supporting Fig. S4). This suggests that Ht2 could significantly increase the promoter activity of OPN.

This finding was further confirmed at the protein level detected by western blot. The OPN protein level of HCC cells transfected with OPN-Ht2 was significantly elevated compared with those transfected with OPN-Ht3 or mock control, but there was no significant difference between the OPN protein expression levels of HCC cells transfected with pGL3-OPN-Ht3 and those with pGL3-Basic plasmid (Fig. 3B).
To further confirm these findings, we also determined the OPN protein levels in 82 primary HCCs with homozygous Ht2 (-443TT genotype) or Ht3 (-443CC genotype) randomly selected from the two cohorts of HCC patients by immunohistochemistry, and found that Ht2 was associated with an increased OPN level in HCCs compared with the homozygous Ht3. Positive OPN staining was found in 21 of 41 (51.2%) HCCs with homozygous Ht2, which was much higher than that of those with homozygous Ht3 (8/41, 19.5%, \( P = 0.003 \)) (Fig. 3C).

**Effects of OPN Promoter Polymorphisms at Locus -443 on HCC Growth and Metastasis.** Then we investigated the effects of SNP at locus -443 of OPN promoter on the *in vitro* invasion and *in vivo* tumor growth and metastasis of HCC cells. In the Matrigel assays, the migrated cell number of HepG2 cells transfected with OPN promoter-reporter construct containing Ht2 (OPN-Ht2) \((39.7 \pm 3.7)\) was significantly higher than those transfected with OPN-Ht3 \((19.0 \pm 4.1)\) or mock control \((17.7 \pm 5.5)\) \((P = 0.020)\). However, no significant difference was found between HepG2 cells transfected with OPN-Ht3 and mock control \((P = 0.854)\) (Fig. 4A,B). This suggests that Ht2 (-443TT genotype), other than Ht3 (-443CC genotype), could significantly increase the invasive ability of HCC cells.

To investigate their effects on *in vivo* tumor growth and metastasis of HCC, HepG2 cell lines transfected with OPN promoter-reporter vectors (OPN-Ht2, OPN-Ht3, or mock control) were subcutaneously implanted into nude mice. The mice were sacrificed and the tumors and lungs were removed at the sixth week after implantation. The mice implanted with HepG2 cells transfected with OPN-Ht2 had markedly larger tumors \((4.05 \pm 0.76 \text{ cm}^3)\) compared with those with OPN-Ht3 or control \((2.70 \pm 0.46 \text{ cm}^3 \text{ and } 2.43 \pm 0.35 \text{ cm}^3, \text{ respectively; } P < 0.05)\) (Fig. 4C,D).

The incidence of pulmonary metastasis was 66.7% \((4/6)\), and the average tumor cluster number of lung metastases per mouse was 66.25 ± 19.16 (grade I, 45.75 ± 11.24; grade II, 16.25 ± 3.78; grade III,
2.75 ± 0.96; grade IV, 1.5 ± 0.58) cells in the OPN-Ht2 group (Supporting Fig. S5), but no lung metastasis was found in the OPN-Ht3 or mock group (P < 0.05) (Fig. 4E).

**Discussion**

It has been strongly suggested that a stepwise, irreversible accumulation of genetic alterations is the responsible driving force for the development and progression of malignancies, and is also being exploited to improve a personalized diagnosis and therapy for cancer. Recent reports of genome-wide association studies (GWAS) and deep sequencing in HCC have provided more evidences that genetic alterations play important roles in tumor occurrence and progression.

The OPN promoter contains a number of potential regulatory elements for transcription factors, such as TATA-like (-27 to -22 nt) and CCAAT-like (-73 to -68 nt) sequences, vitamin-D-responsive (VDR)-like motifs (-1892 to -1878 nt and -698 to -684 nt). More than 10 SNPs have been identified in the OPN promoter; some appear to be risk factors for hepatitis activity and sustained virological response (SVR) in Japanese patients with chronic hepatitis C. However, little is known about the impact of OPN polymorphisms on cancer progression.

In this study we characterized the allelic architecture of the OPN promoter and identified SNPs at four loci: -1748, -616, -443, and -155, and the three most common haplotypes (Ht1, Ht2, and Ht3) of OPN promoter region in HCCs. It is very interesting that only one nucleotide difference at locus -443 was found between the two major haplotypes (Ht2 and Ht3). Furthermore, -443TT/TC genotypes were more frequently found, which are consistent with the previous reports in chronic hepatitis C patients from Egyptian and Japanese patients and -443TT has been regarded as a significant variable reflecting hepatitis activity in patients with chronic hepatitis C. This suggests that the SNP at locus -443 might be more important in the progression of liver diseases, including HCC.

Another important finding of this study is that only SNPs at locus -443 and their related haplotypes were closely associated with HCC prognosis. The -443TT/TC genotypes and Ht2 significantly increase the probability of HCC recurrence and result in a poorer prognosis, whereas the patients with -443CC genotype or...
Ht3 have a lower probability of tumor recurrence and longer survival. Uni- and multivariate Cox regression analyses indicated that the -443 genotypes and related haplotypes were independent prognostic factors for HCCs. These findings were further confirmed in a larger independent validation cohort of HCC patients. This suggests that, besides the OPN expression level, the OPN promoter polymorphisms, particularly at -443, can also be a part of the genetic variations that underlie the phenotypic variations seen in individual outcomes in cancer, and might serve as a powerful predictor for HCC prognosis. However, the -443TT/TC genotypes are much more prevalent than the -443CC genotype in HCCs, and this might be one reason their predictive power is not very strong and may also be a limitation for their application in clinical practice.

More important, this prognostic value of SNP at locus -443 still remains significant in patients with small HCCs (≤5 cm in diameter). It is well known that tumor size is one of the significant prognosis factors for cancers, including HCC. In clinical practice, patients with a solitary HCC ≤5 cm in diameter are usually considered adequate candidates for surgical resection, provided they have well-preserved liver function, appropriate geographic distribution of the tumor, and good performance status. These patients are thought to have a relatively lower risk of vascular invasion and intrahepatic metastasis, which could improve
the surgical outcome. However, some patients with small HCC still have a poor prognosis in clinical practice, presenting clinicians with a major challenge in the prognosis of these patients. In this study we demonstrated that OPN promoter polymorphisms could also statistically affect the prognosis of the patients with a small HCC; SNP at locus -443 and related haplotypes could be helpful to identify those patients with a worse prognosis. It would be useful to provide supplemental information for the prediction of patient outcomes after operation of different subgroups and better selection of therapeutic strategies.

To explore how the OPN promoter polymorphisms affect HCC prognosis, we first analyzed the functional effects of SNP at locus -443 or the related haplotypes on the promoter activity and expression levels of OPN in HCC, and then evaluated their influence on the proliferation and metastasis of HCC cells. We found that the -443T allele could increase transcriptional activity and expression level of OPN compared with the protective -443C allele. Because SNP at locus -443 is located just upstream of the cis-acting enhancing element of human OPN, such SNPs can be sure to affect OPN expression. Perhaps the presence of a strong transcription factors site, as in the -443T/C allele, within the context of OPN promoter could change the balance of the basic transcription complex toward enhanced transcription and expression of OPN. Some transcription factors such as MYT1 and c-Myb have been reported to bind OPN promoter at position -443.

We noticed that although Ht3 could also increase OPN promoter transcriptional activity, it did not significantly change the OPN protein levels. The reason for this discrepancy is not clear. There might be some unknown factors regulating the protein levels of OPN with Ht3, and the transcript variations are not always observed in the corresponding protein levels.

Increased OPN levels have been demonstrated to be related to tumor progression and dismal prognosis of many human cancers, including HCC. This implies that the -443TT genotype of the OPN promoter affects HCC prognosis by up-regulating the expression of OPN. However, these findings are different from a previous report in which significantly higher levels of OPN mRNA were found in melanoma metastases with homozygous -443C compared with those with heterozygous (-443T/C) or homozygous -443T. The real reason for this discrepancy is not clear. It might be due to the different tumor backgrounds of the patients tested. In addition, the haplotypes of polymorphism instead of single SNP, which has been proven to produce more accurate results in several common diseases. Furthermore, two large cohorts of patients were used in the present study. This could increase the reliability of the study.

We also found that Ht2 (-443T allele) could lead to significant increases in both in vitro invasion and in vivo tumor growth and lung metastasis of HCC cells. In view of the above evidence, we propose that Ht2 of OPN promoter in primary tumors may promote the progression and metastasis of HCC by regulating the OPN expression. This is consistent with our previous finding that OPN plays important roles in tumor growth, invasion, and metastasis of HCC, and is helpful in understanding the mechanisms by which OPN regulates the proliferation and metastasis of HCC. This provides an interesting possibility to develop a potential therapeutic strategy to block the OPN function and control HCC metastasis.

In summary, we identified an SNP at locus -443 and its related haplotypes (Ht2, Ht3) in the OPN promoter region as novel prognostic factors for HCC. Ht2 or -443TT genotype could significantly increase the promoter activity and expression level of OPN, and lead to an obvious increase of tumor growth and lung metastasis of HCC cells. This indicates that the genetic variation at locus -443 of the OPN promoter plays an important role in the regulation of OPN expression and cancer progression of HCCs, which is a novel determinant and target for HCC metastasis and prognosis. This provides further evidence for the important role of OPN in HCC progression and new insight into the regulatory mechanism governing OPN expression, which may be a novel therapeutic target for HCC.

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