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Original Article

Osteopontin promoter polymorphisms at locus -443 are associated with metastasis and poor prognosis of human intrahepatic cholangiocarcinoma in Chinese population

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Abstract: Purpose: Osteopontin (OPN) is known to be a secreted adhesive glycoprotein. Role of OPN in human intrahepatic cholangiocarcinoma (ICC) has not been well understood. This study explored whether genetic variations in the osteopontin gene are associated with ICC risk, progression and metastasis. Material and methods: 260 patients with stages I to IV between 2008 and 2013 were recruited in this study and same number healthy persons were used as control. OPN-66 T/G, -156 G/GG and -443 C/T variants were genotyped using DNA from blood lymphocytes. Chi-square test and a Fisher's exact test were used to analyze the genotype distribution between healthy subjects and patients, and further its distribution among TNM stages and incidence metastasis in patients. Results: For the variant at nt- 443 (CC), there was a significant difference between the number of patients with stage IV and those with all other stages of ICC ($P < 0.01$). Patients with -443 (CC) variant had significant higher incidence of lymph and distant metastasis development compared to other genotypes. For the variant at nt- 443 (CT), there was a significant difference between the number of ICC patients with stage III + IV and those with stage I + II ($P < 0.01$). The survival rates for ICC patients with the C/C genotype were significantly lower than for patients with the other two genotypes (C/T, T/T). Conclusion: OPN -443 C/T polymorphism is a potential predictive marker of metastasis and poor prognosis in ICC patients.

Keywords: Osteopontin, intrahepatic cholangiocarcinoma, genetic variants, metastasis

Introduction

Intrahepatic cholangiocarcinoma (ICC) is the second most frequent form of primary hepatic malignancy in adults after hepatocellular carcinoma [1]. Although significant progress in the diagnostic and surgical approaches has been achieved, the survival rates for patients with ICC remain unfavorable [2-4]. Despite recent advances, the molecular or genetic mechanisms involved in the progression of ICC still remain poorly understood. Thus, to develop effective individualized treatments based on molecular classification are pivotal to improve the prognosis of ICC patients. Although many biomarkers have been evaluated for their prognostic significance in ICC, none of these have been proven to be a predictive power of the prognosis with high specificity and sensitivity for ICC.

Osteopontin (OPN) is a secreted non-collagenous, sialic-acid-rich, chemokine-like extracellular matrix (ECM) protein [5]. OPN binds to αvβ integrins and receptors of the CD44 family to promote cell adhesion, chemotaxis, ECM degradation, angiogenesis, prevention of apoptosis, and indolent tumor growth [6]. Moreover, it plays a crucial role in determining the oncogenic potential of various cancers, contributing to tumor invasion and metastasis [7-10]. Previously lots of studies have demonstrated that OPN is one of the highest overexpressed genes in hepatocellular carcinoma (HCC) and it has been shown the expression of OPN correlates with earlier recurrence, poorer prognosis and metastasis in HCC [11, 12]. However, about its role in the ICC, there are few controversy reports so far. Based on our best knowledge, there are three reports about the OPN expres-

Table 1. Clinicopathologic characteristics of patients with ICC and healthy controls

Characteristics	No. of patients or controls		*P*
	Case (n)	controls (n)	
No.	260	260	
Age, y			> 0.05
Median	57.2	56.3	
Range	24-81	23-87	
Gender			> 0.05
Male	149	147	
Female	111	113	
Alcohol abusing			< 0.01
Never	170	218	
Former	26	13	
Current	64	29	
Location		-	-
Hilar	89	-	
Peripheral	171	-	
Histology		-	
Differentiated	85	-	
Undifferentiated	175	-	
UICC stage grouping (sixth edition)		-	
I	49	-	
II	67		
III (A-C)	115	-	
IV	29	--	
Lymph node		--	
Positive	160	-	
Negative	100	-	
Tumor size (cm)		-	
≤ 4	138	-	
> 4	122	-	
Distant metastasis			
Yes	29		
No	231		

sion condition in ICC. Terashi et al observed a correlation between low OPN levels and tumor aggressiveness [13], whereas another group found high levels of the glycoprotein in ICC in a rat model system [14]. More interestingly, Holger G Hass identified osteopontin as the most consistently over-expressed gene in intrahepatic cholangiocarcinoma by oligonucleotide microarray and real-time PCR analysis from surgical specimens [15]. Therefore, it deserves further study about whether OPN is a potential prognostic marker and target for anticancer treatment in ICC.

Single nucleotide polymorphisms at oncogene or tumor suppressor gene promoter region sometimes can influence the gene expression significantly, thus causing significant difference on cancer occurrence, metastasis and prognosis, et al. These genetic variants may be good biomarkers for predict prognosis for cancer patients. Previous study has confirmed that OPN promoter polymorphisms at locus -443 significantly affect the metastasis and prognosis of human hepatocellular carcinoma. However, there are no relative reports about the relationship between OPN polymorphisms with ICC currently. In the present study, we recruited 260 ICC patients and 260 cancer-free control, aim to investigate whether OPN promoter polymorphisms -66 T/G, -156 G/GG, and -443 C/T genotypes affect the occurrence, metastasis and prognosis of ICC patients.

Patients and methods

Patients

This study comprises a total of 260 primary ICC patients who had not undergone preoperative treatment (with a mean age of 59 ± 11.2 years) seen at the Clinic of General Surgery and Transplantation of the 301 Hospital, between August 2008 and June 2013. The diagnosis of ICC was based on histology obtained by preoperative or intraoperative biopsy or by examination of resected liver specimens. Patients with gallbladder carcinoma or mixed hepatocellular carcinoma/ICC and cirrhosis were excluded from this study. Available data included age, sex, tumor location, tumor size, histological differentiation, tumor stroma, vascular invasion (portal vein, hepatic vein, hepatic artery, and bile duct invasion), lymphatic permeation, perineural invasion, intrahepatic metastasis, and lymph node metastasis (**Table 1**).

Healthy control group consisted of a random sample of 260 age-matched and sex-match-

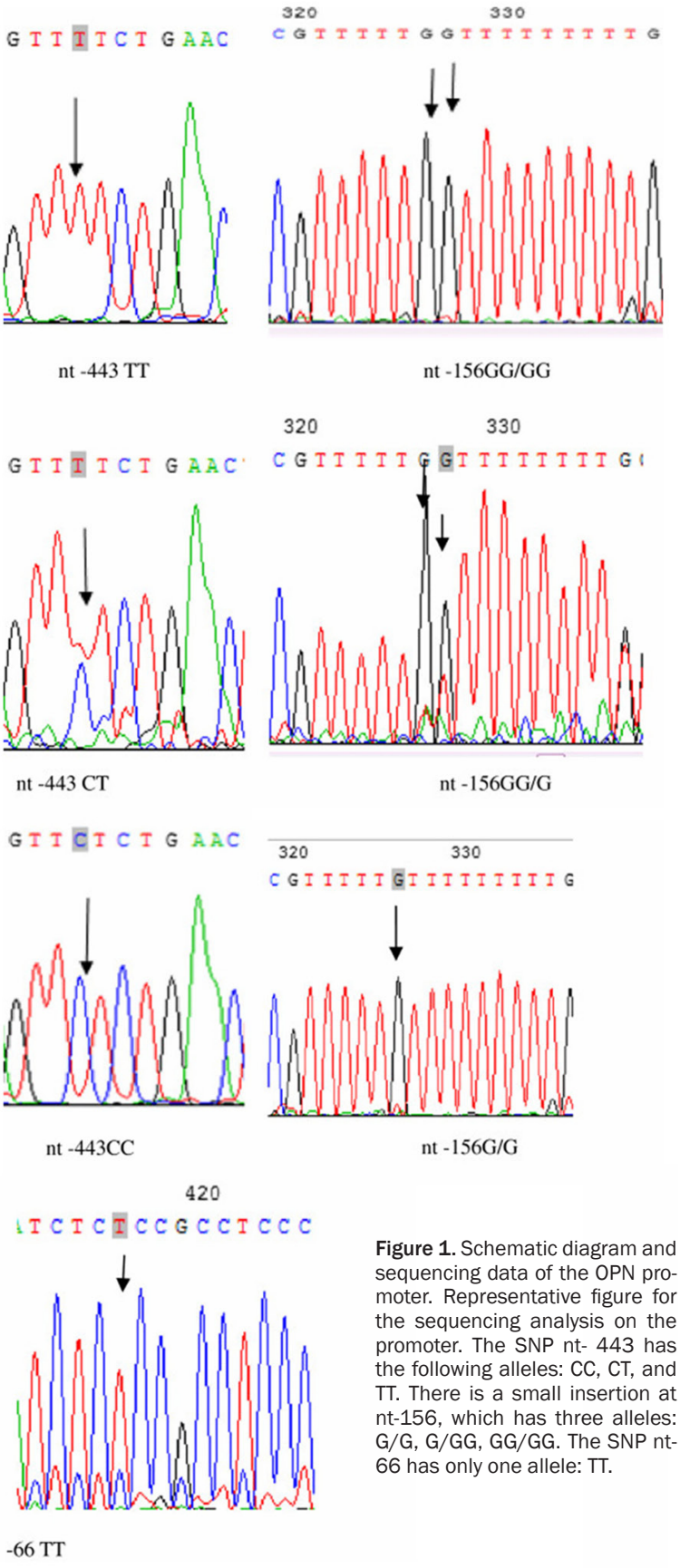

Figure 1. Schematic diagram and sequencing data of the OPN promoter. Representative figure for the sequencing analysis on the promoter. The SNP nt- 443 has the following alleles: CC, CT, and TT. There is a small insertion at nt-156, which has three alleles: G/G, G/GG, GG/GG. The SNP nt-66 has only one allele: TT.

ed ethnic Han Chinese from Beijing.

All the participants agreed to participate in this study and had adequate blood DNA for genotyping and all had complete follow-up and clinical information. There was no significant difference in the distribution of demographic information between patients enrolled and patients who did not. Written informed consent was obtained from each participant for the use of their DNA and clinical information. The study was approved by the Institutional Review Board of 301 Hospital, Beijing, China.

SNP genotyping

Genomic DNA was extracted from 5-mL blood sample that was collected from each patient and healthy subject upon recruitment. The OPN-66 T/G, -156 G/GG (rs17-524488), and -443 C/T (rs11730582) variants were genotyped by direct sequencing of the sense and antisense strands following polymerase chain reaction (PCR) amplification of the promoter regulatory region -473 to -3 (forward primer 50-CAA GCT ACT GCA TAC TCG AAA TCA CA-30; reverse primer 50-ACA ACC AAG CCC TCC CAG AAT TTA-30), as previously described [16]. PCR was performed using 50 ng DNA as a template under the following conditions: 95°C for 10 min, then 36 cycles of 94°C for 30 s, an annealing temperature for 60 s, and 72°C for 60 s, with a final extension at 72°C for 15 min. After affinity membrane purification using the QIAquick Gel Extraction kit (Qiagen, Carlsbad, CA, USA),

Table 2. Comparison of OPN promoter between ICC patients and healthy controls

	controls	Patients		ICC					
	n	n	*P*	LN (+)	LN (-)	*P*	DM (-)	DM (+)	*P*
-66 T/G									
TT	251	256	1.00	158	99	1.00	228	28	1
TG	9	4	0.261	2	1	0.637	3	1	0.537
-156									
G/G	107	111	1.00	53	35	1.00	101	13	1
G/GG	110	101	0.563	74	45	0.775	92	10	0.826
GG/GG	43	48	0.804	33	20	0.860	38	6	0.786
-443									
TT	114	120	1	49	47	1.00	111	8	1.000
CT	115	111	0.709	63	38	0.116	102	9	0.803
CC	31	29	0.773	48	15	0.002	17	12	< 0.001

Note: LN: Lymph node metastasis; *P* value was calculated by chi-square test and a Fisher's exact test. DM: distant metastasis.

Table 3. The distribution of genotypes for TNM stages among ICC patients

	The TNMs of ICC				
Genotypes	I	II	III	IV	*P*
-66					0.603
TT	48	67	113	28	
TG	1	0	2	1	
-156					0.730
G/G	21	34	46	10	
G/GG	18	24	47	12	
GG/GG	10	9	22	7	
-443					< 0.001
TT	36	35	41	8	
CT	11	28	63	9	
CC	2	4	11	12	

Note: *P* value refers to significance value among all the groups on SNP, and was calculated by person chi-square test.

the PCR products were subjected to cycle sequencing with the respective forward and reverse primer using an automated ABI 3100 DNA sequencer by GeneCore Bio Technologies (Shanghai China). A 15% blind, random sample of study subjects was genotyped twice by different persons and the reproducibility was 100%.

Statistical analysis

Statistical analysis was performed using SPSS 18.0 software. Quantitative variables departing from the normal distribution, including age, gender and alcohol abusing status were summarized. Comparison of age between cases and controls was assessed using an independent Student's t-test. Comparison of gender, alcohol abusing status and genotype frequencies between cases and controls was assessed using a chi-square test and a Fisher's exact test. Survival was calculated by the Kaplan-Meier method. All probability (*P*) values were two-tailed and statistical significance was indicated as $P < 0.05$.

Results

Patient characteristics and clinical outcomes

This study recruited 260 patients with ICC and 260 healthy controls. The baseline clinical characteristics of patients are summarized in **Table 1**. There were no significant differences in terms of distribution of age and gender, but significant on alcohol abusing status, suggest alcohol abusing is one of risk factors. Clinicopathologic characteristics of the patients and controls are shown in **Table 1**.

SNPs in the promoter region of human OPN gene

Direct sequencing of DNA fragments between nt-473 and nt-3 in patients and age- and gender-matched controls revealed 3 SNPs in the OPN promoter, located at nt -156 [GG/GG homozygotes, GG/G-(deletion) heterozygotes, G-/G- homozygotes], nt- 443 [CC homozygotes, CT heterozygotes, TT homozygotes], and nt -66 (**Figure 1**), as shown in **Table 2**. There was no significant difference in the distribution of these SNPs (nt -66, -156, -443) between patients and controls. The distribution of genotypes for TNM stages in ICC patients is shown

Table 4. The genotype distribution of nt- 443 in the OPN promoter by ICC TNM stage

	The TNM stages of ICC					
Genotypes	I + II	III + IV	*P*	I + II + III	IV	*P*
-443						
TT	71	49	1.000	111	8	1.000
CT	39	72	< 0.001	102	9	0.803
CC	6	23	< 0.001	17	12	< 0.001

in **Table 3**. However, regarding tumor-node-metastasis TNM stages, we found that for the SNP at nt- 443, there was significant difference on distribution of three genotypes among four stages ($P < 0.001$, **Table 3**). Among patients with the CT genotype, there was a significant difference between patients with stages I + II and stages III + IV ($P < 0.01$), data was shown in **Table 4**. Similarly, among patients with the CC genotype at nt- 443, there was a significant difference between patients with stages III + IV and stages I + II ($P < 0.01$) and between stages IV and combination of stage I to stage III ($P < 0.01$; **Table 4**). There were no significant differences among the TNM stages and the other two SNPs (nt -66 and nt -156) of the OPN promoter.

Effect of SNPs on lymph and distant metastasis

As shown in **Tables 2** and **3**, there were total 29 patients who had CC genotype at nt- 443, among them, 12 cases were at stage IV and had distant metastasis. By compared with TT genotype, it demonstrated that CC genotype at nt- 443 might significantly increase the risk of development of distant metastasis ($P < 0.01$). We also found that significant association between the -443 genotypes in the OPN promoter and lymph node metastasis, type CC had more risks to develop lymph node metastasis (**Table 2**).

Associations between genotypes in the OPN promoter region and survival

Kaplan-Meier estimates of different genotypes at nt- 443 in the OPN promoter are shown in **Figure 2**. The survival rates for patients with the C/C genotype were significantly lower than the survival rates for patients with the other two genotypes (C/T, T/T). There were no significant associations between survival and genotypes at the other sites (nt -156 and nt -66).

Discussion

Based on my knowledge, it is first time to report the relationship between OPN polymorphisms and risk of ICC patients. Lots of evidence suggests that OPN plays a role in the regulation of tumor metastasis and that OPN expression is particularly high in metastatic tumors [17-19]. OPN is overexpressed in cancers that have a high propensity for forming distant metastases. Moreover, high OPN expression in the primary tumor is associated with early metastasis and poor clinical outcome in hepatocellular carcinoma and other cancers [16, 17, 20-23].

There is arguing about the role of OPN in ICC patients. Dr Tomohiro Iguchi selected OPN expression as a risk factor in the split of the survival tree model in ICC and demonstrated that lower expression of OPN was the best predictor of the patients' prognosis [1]. In the present study, we focused on the association of these SNPs with TNM stages of ICC, especially for distant metastasis. Although the distribution of genotypes (CC) at nt- 443 in the OPN promoter was not significantly different between ICC patients and healthy controls, there were significant difference in the distribution of genotypes between patients with stage IV and other stage ICC (**Table 4**).

Recent study proved that the haplotype -443C/-156 G/-66 T of OPN gene is associated with significantly enhanced promoter activity compared to five other allelic variants tested [24]. A recent study on melanoma metastases found that those homozygous for the -443C allele expressed significantly higher levels of OPN mRNA compared to those that were either heterozygous (CT) or homozygous for the -443 T allele [25]. Transcription factor c-Myb binds to the region of the OPN promoter in an allele-specific manner and induces enhanced activity of the -443C compared to the -443 T OPN promoter [26]. Taken together, these data suggest that the variation at nt- 443 in the OPN promoter plays a role in GC progression and metastasis, especially for the CC genotype at nt- 443 in the OPN promoter. Whether the polymorphisms of OPN are related to expression of OPN in cancer patients remain unknown. Over-expression of OPN was found in ICC samples in a previous study [15], and the alteration of the -443 T → C

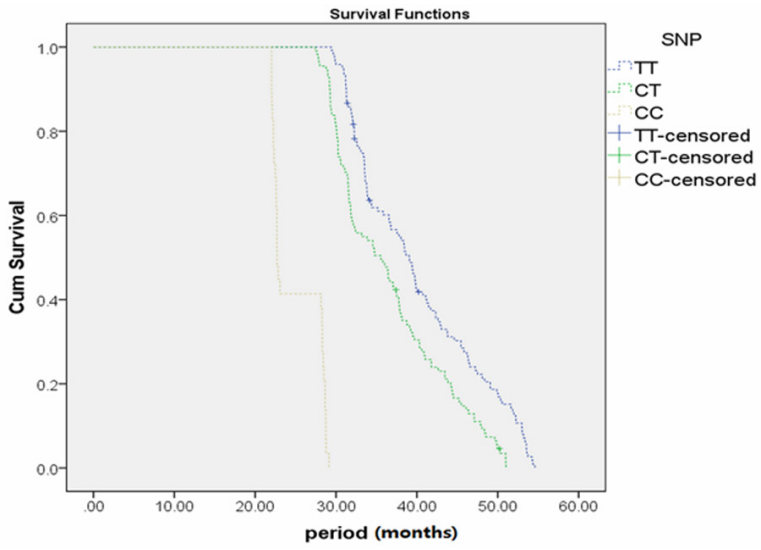

Figure 2. Kaplan-Meier survival is significantly lower in ICC patients with the C/C genotype as compared to the other two genotypes at nt -443 in OPN promoter.

promoter region could significantly increase the promoter activity by Dual Luciferase Reporter Assay System [16].

In the present study, we found that the CT genotype at nt- 443 in the OPN promoter showed significant differences between stages III + IV and stage I + II ICC, but no significant difference between stage IV and sum of other stages of ICC (**Table 4**); and for the CC genotype, there was significant difference between stage IV and other single stages or combination of any other stages. The main reason for this may be due to the limited number of patients in CC type subgroups. It is also possible that the CC genotype has more enhanced transcription activity of the region of the OPN promoter compared to CT genotypes [25]. Among total 29 CC genotype patients, 12 patients were diagnosed as distant metastasis, it is extremely high, but there is no significant difference on the ratio of CC type between ICC patients and healthy controls. The main reason for this, we hypothesize that OPN is a not key factor for initiating ICC, but once the carcinogenesis occurred, OPN will enhance this process effectively, especially for distant metastasis and lymph metastasis, which is consistent with previous study [27]. However, the further study is needed to investigate this hypothesis. Meanwhile, the current study also provides another evidence to suggest over-expression of OPN may correlate with the poor prognosis of ICC, which is different from Terashi's report.

There are also some drawbacks in the present study, one of them is because all the subjects are Chinese individuals, the results should be interpreted with caution and need to be confirmed in larger and ethnically divergent population samples. On the other hand, the number of stage IV patients in the current study is not high enough, so the large-population research is needed to make stronger conclusion about the association between distant metastasis formation and -433 polymorphisms.

In summary, -443 C/T of OPN is a potential biomarker for predicting prognosis of ICC especially for lymph and distant metastasis.

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Disclosure of conflict of interest

None.

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