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Original Paper

OPN -443C>T Genetic Polymorphism and Tumor OPN Expression are Associated with the Risk and Clinical Features of Papillary Thyroid Cancer in a Chinese Cohort

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Key Words
Papillary thyroid cancer • Osteopontin • Polymorphism • Risk

Abstract
Aim: to test the possible association between the polymorphism of Osteopontin (OPN) gene with the risk and the clinical features of papillary thyroid cancer (PTC). ***Methods:*** A total of 363 PTC patients and 413 healthy controls were enrolled. OPN expressions in tumor tissue were detected by immunohistochemistry. OPN gene polymorphisms, namely, -66T>G (rs28357094), -156G>GG (rs17524488), and -443C>T (rs11730582), were determined. ***Results:*** We observed that the PTC patients had significantly higher rates of -443TT genotypes than controls (P<0.001). The multivariate logistic regression analysis showed a significantly increased risk for PTC for the -443CC genotype compared with the -443TT genotype (adjusted OR= 4.312, 95%CI: 2.747 -6.987, adjusted P < 0.001). OPN protein was not expressed in normal thyroid tissues while tumor samples from PTC patients were shown to have high expressions of OPN. Also, we found that the high OPN expressions were significantly more prevalent in -443CC carriers than TT carriers (P <0.001). Both the CC carriers and OPN expression were closely associated with the cervical lymph node metastasis and angiolymphatic invasion of PTC. ***Conclusion:*** This study provides evidence to support the connection between the OPN genetic polymorphism and tissue expression with risk as well as the invasiveness of PTC.

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Introduction

Thyroid tumors are the most common malignancies of the endocrine system. They account for roughly 1% of all new malignant diseases and are the sixth most common malignancy diagnosed between the ages of 20 and 49 years [1, 2]. Papillary thyroid cancer

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(PTC) account for the majority of thyroid tumors [3]. Radiation exposure is the strongest known risk factor for PTC [4]. However, examining other risk factors ,such as genetic factors, could help to advance in the treatment and prevention of PTC, which has a good prognosis if detected early and treated appropriately [5, 6]. In addition, family history studies have suggested that the incidence of thyroid cancer may have a hereditary predisposition, with the reported relative risk estimates of 3- to 4-fold or higher [7, 8] when examining familial PTC occurrence. Some studies reported the close association between some gene variant and risk of PTC, suggesting that the genetic polymorphism may be used as a molecular marker to screen subjects at high risk [9-11] However, the ideal genetic marker for PTC incidence is still undetermined [12].

Osteopontin (OPN) is a matricellular protein that participates in a wide range of physiological and pathologic processes, including tumorigenesis and metastasis [13-16]. Recent studies showed that OPN is involved in the tumorigenesis of breast cancer [17-19], hepatic carcinoma [20], gastric cancer [21]. The role of OPN in thyroid cancer received much attention in recent years. *In vitro* studies revealed that the OPN plays a pivotal role in the expression of the mitogenic and invasive phenotype of RET/PTC-transformed thyroid cells [22]. Nearly all normal thyroid samples were negative for OPN; however, thyroid adenomas were OPN positive [23]. Clinically, OPN is over-expressed in human papillary thyroid carcinomas and enhances thyroid carcinoma cell invasiveness. The prevalence and intensity of OPN staining were significantly correlated with the presence of lymph node metastases and tumor size [24].

The expression of OPN was significantly influenced by its genetic polymorphisms of the promoter [25]. Several polymorphisms in the human OPN encoding gene have been identified in different populations, of which the −156 GG>G genotype and −443 T>C polymorphisms were the most studied. Genetic polymorphisms in the OPN promoter also increases the risk of distance metastasis and death in Chinese patients with gastric cancer [26]. OPN gene polymorphisms are associated with susceptibility and clinicopatholigical characteristics of cervical cancer in a Chinese cohort [27]. OPN promoter polymorphisms at locus -443 significantly affect the metastasis and prognosis of human hepatocellular carcinoma [28]. However, no study regarding the role of OPN in PTC has been reported.

Based on the important significance of OPN in thyroid cancer risk, we postulated that the polymorphism of OPN gene might be associated with the risk and the clinical features of PTC. Thus, we performed current case-controls study to testify the hypothesis.

Materials and Methods

Patients and controls

PTC patients involved with this study were enrolled at our hospital between July 2006 and December 2011. PTC was confirmed by pathologic examination. Specimens determined to be benign tumors, follicular variants, and diffuse sclerosing were excluded. Eventually, 363 PTC patients were selected to participate. A total of 413 healthy control subjects were enrolled from a general health check-up program without clinical evidence of cancers, thyroid diseases, or any other chronic or severe conditions. This study was approved by the ethics review board of the Medical Research Institute of our hospital. Written informed consent was obtained from each subject prior to study entry.

OPN gene polymorphisms

DNA was extracted from peripheral whole blood using a Qiagen DNA Isolation Kit (Qiagen, Valencia, CA, USA). Three single nucleotide polymorphisms on the promoter region of *OPN* gene, including -66T/G (rs28357094), -156G/GG (rs17524488), and -443C/T (rs11730582), were determined using TaqMan 5′ allelic discrimination assay. It was performed using a commercially available kit Assays-on-DemandTM SNP genotyping products (Applied Biosystems, Foster City, CA). SNP amplification assays were used according to the manufacturer's instructions. In short, 10 ng of sample DNA in 25 μL of reaction solution containing 12.5 μL of the 2× TaqMan® Universal PCR Mix (Applied Biosystems), and 1.25 μl of pre-developed assay

Table 1. shows the association between the CFTR SNPs and the clinical characteristics of PTC

Characteristics	PTC(n=363)	Controls(n=413)	P
e (years)	38.6±2.1	38.4±4.3	0.254
Gender			
Male	143	204	0.025
Female	220	209	
Smoke status			
Non-smokers	271	126	0.225
Smoker	92	287	
Radiation exposure history			
Yes	51	2	<0.001
No	312	411	
Cancer size (n)			
<1cm	289		
>1cm	74		
Number of tumors (n)			
Unifocality	211		
Multifocality	152		
Location of cancer (n)			
One lobe	195		
Both lobes	168		
Extra thyroidal invasion (n)			
Absent	188		
Present	175		
Cervical lymph node metastasis (n)			
Absent	293		
Present	70		
Angiolymphatic invasion (n)			
Absent	300		
Present	63		
Distant metastases			
Absent	348		
Present	15		
Extent of surgery			
One side total+ contralateral resection	261		
Two sides total resection	102		
Treatment			
Radio-iodine + surgery	66		
Surgery	297		

reagent from the SNP genotyping product containing two primers and two MCB-Taqman probes. Reaction conditions consisted of pre incubation at 50 °C for 2 min, at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Amplifications were performed in an ABI Prism® 7500 Sequence Detection System (Applied Biosystems). To re-confirm the results of genotypes, we randomly selected 25 patients and 25 controls (10% of the study subjects) for re-genotyping by direct sequencing; the results were 100% identical.

Immunohistochemistry

Formalin-fixed and paraffin-embedded 4- to 5-μm-thick tumor sections were deparaffinized, placed in a solution of absolute methanol and 0.3% hydrogen peroxide for 30 min, and treated with blocking serum for 20 min. The slides were incubated overnight with anti-OPN monoclonal antibodies, with biotinylated anti-IgG, and finally with premixed avidin-biotin complex (Vector Laboratories, Inc., CA). The immune reaction was revealed with 0.06 mmol/liter diaminobenzidine (DAB-Dako, DakoCytomation, Carpinteria, CA) and 2 mmol/liter hydrogen peroxide. The OPN immunostaining was mostly localized in the cytoplasm. Considering the percentage of OPN immune-positive tumor cells, a score of 1 was given when ≤10% of cells were positive; 2 when 10–50% of cells were positive and 3 when ≥50% of cells were positive. Signal intensity

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Table 2. The genotype distributions and allele frequencies of OPN polymorphisms in PTC and control subjects

OPN genotype		PTC		Control		adjusted OR	95%CI		adjusted P
		N	%	N	%				
-156/G/GG	GG	104	25%	100	21%	1.000			
	GGG	187	44%	219	46%	0.821	0.436	1.167	0.253
	GGGG	72	17%	94	20%	0.736	0.478	1.143	0.147
	G	395	47%	419	44%	1.000			
	GG	446	53%	532	56%	0.889	0.712	1.134	0.221
-66T/G	TT	99	25%	114	25%	1.000			
	TG	167	42%	191	42%	1.007	0.787	1.465	0.736
	GG	97	24%	108	24%	1.034	0.724	1.753	0.887
	T	365	46%	419	46%	1.000			
	G	431	54%	490	54%	1.010	0.898	1.276	0.853
-443C/T	TT	73	20%	164	40%	1.000			
	CT	171	47%	187	45%	1.854	0.955	2.901	0.054
	CC	119	33%	62	15%	4.312	2.747	6.987	<0.001
	T	317	44%	515	62%	1.000			
	C	409	56%	311	38%	2.137	1.675	2.456	0.031

was scored as negative (0), weak (1), moderate (2) and strong (3). Both scores were multiplied and the resulting score was used to categorize OPN expression as low (0–6) and high (>6) expressions [29].

Statistical Analyses

$\chi 2$ tests were used to compare genotype frequency and demographic distributions between cases and controls. Multiple logistic regression analyses were used to evaluate if each SNP was independently associated with PTC when adjusted for the potential confounding effects of important clinical variables. The odds ratios (OR) and 95% confidence intervals (CIs) were calculated. All analyses were performed by using SPSS software (Statistical Package for the Social Sciences, version 16.0, SPSS Inc, Chicago, IL, USA).

Table 3. OPN expression status between PTC and normal tissues

	PTC	Control	P
High OPN expression	68	0	<0.001
Low OPN expression	32	12	

Table 4. The OPN genotype and OPN expression in PTC patients

		High OPN		Low OPN		P
-443C>T	TT	14	21%	13	41%	<0.001
	CT	26	38%	15	47%	
	CC	28	41%	4	13%	

Results

The clinical characteristics of PTC cases and controls are listed in Table 1. The age and smoking status were similar between PTC and controls. The age and smoking status were similar between two groups. However, females and those with radiation exposure history were more numerous in the PTC group than in the control group (P=0.025 and P>0.001, respectively).

Table 2 describes the genotype distributions and allele frequencies of OPN poly-morphisms in PTC and control subjects. The genotype frequencies for all polymorphisms did not differ significantly from those expected under Hardy–Weinberg equilibrium (both P>0.05). The genotype frequencies and allele frequencies at OPN -156 G>GG and -66 T>G were similar in PTC and control subjects. In contrast, the genotype of -443 C>T was significantly different between two groups. The PTC patients had significantly higher rates of CC genotypes than controls (33% vs.15%, P<0.001). Accordingly, the -443C allele frequencies were higher in PTC patients than controls (56% vs. 38%, P=0.031). In order determine the association of OPN gene polymorphism with PTC risk, we performed the multivariate

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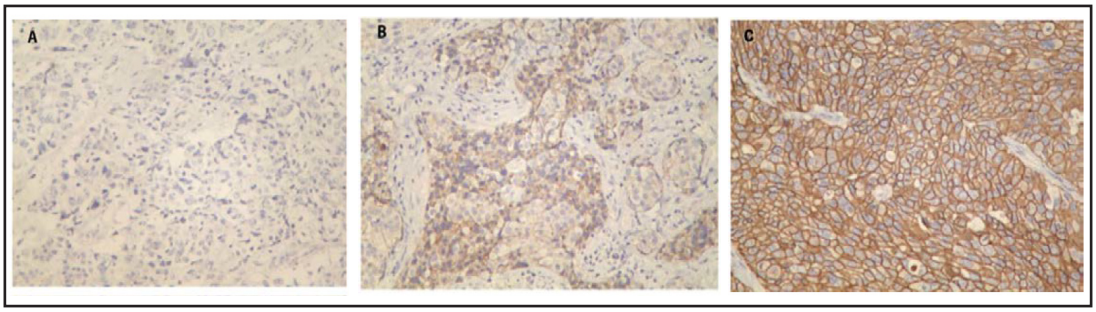

Fig. 1. A: No OPN expression in the non-tumor thyroid tissue samples from a patients with thyroiditis. B: Low OPN expression in PTC tumor tissues from a PTC patient with -443 TT genotype. C: High OPN expression in PTC tumor tissues from a PTC patient with -443 TT genotype.

logistic regression analysis with adjustment with age, sex and smoking status, family history, and radiation exposure history. Our data showed a significantly increased risk for PTC for the -443CC genotype compared with the -443TT genotype (adjusted OR= 4.312, 95%CI: 2.747 -6.987, adjusted P < 0.001) after adjustment.

The OPN expressions in PTC tumor samples were determined by immuhistological staining. We collected 100 samples from surgical treatment or fine needle biopsy and obtained non-tumor thyroid tissue samples from 12 patients that underwent thyroid resection due to thyroiditis. We found no OPN expression in all the non-tumor thyroid tissue samples and observed that OPN was highly expressed in 68 of 100 samples from PTC tumor tissue. A significant difference of OPN expression status between PTC and normal tissues was noted (P<0.001, Table 3). The typical OPN expressions are showed in Figure 1.

We next analyzed the OPN genotype and its protein levels in PTC patients. We observed that the high OPN expression was more frequent in samples from -443CC carriers than TT carriers (41% vs. 21%, P <0.001, Table 4).

Table 5. The correlation between the OPN polymorphisms and PTC clinical features

-443C>T	Tumor size		OR	95%CI		P
	<1 cm	>1 cm				
TT	54	19	1.000			
CT	143	28	1.797	0.927	3.481	0.080
CC	92	27	1.199	0.610	2.358	0.599
	Tumor number					
	Unifocality	Multifocality				
TT	37	36	1.000			
CT	100	71	1.370	0.790	2.376	0.293
CC	74	45	1.600	0.887	2.886	0.117
	Tumor location					
	One lobe	Both lobes				
TT	30	43	1.000			
CT	89	82	1.556	0.894	2.708	0.117
CC	76	43	2.133	1.093	4.606	0.052
	195	168				
	Extra thyroidal invasion					
TT	Presence	Absence				
CT	40	33	1.000			
CC	91	80	0.938	0.541	1.627	0.205
TT	57	62	0.758	0.423	1.361	0.354
CT	188	175				
	Cervical lymph node metastasis					
	Presence	Absence				
TT	51	22	1.000			
CT	134	37	1.562	0.842	2.900	0.156
CC	108	11	4.235	1.909	9.395	<0.001
	Angiolymphatic invasion					
	Presence	Absence				
TT	53	20	1			
CT	141	30	1.774	0.928	3.390	<0.001
CC	106	13	3.077	1.422	6.660	0.003

We next analyzed the correlation between the OPN polymorphisms and the clinical features. We found that the -443 C>T was not associated with tumor size, tumor number, or tumor location, but was significantly related to the cervical lymph node metastasis and angiolymphatic invasion. -443CC carriage was significantly associated with higher risk for cervical lymph node metastasis and angiolymphatic invasion compared with -443 TT carriage (Adjusted OR=4.235, P<0.001 and OR=1.422, P=0.003, respectively, Table 5).

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The correlation between the tumor tissue OPN expression and PTC clinical feature is showed in Table 6. The OPN high expression was related to the cervical lymph node metastasis and angiolymphatic invasion as well.

Table 6. The correlation between the tumor tissue OPN expression status and PTC clinical features

OPN expression	Tumor size		OR	95%CI		P
	>1 cm	<1 cm				
Low	21	11	1			
High	50	18	1.455	0.004	528.912	0.901
Tumor number						
	Unifocality	Multifocality				
Low	17	15	1			
High	44	24	1.618	0.004	617.585	0.874
Tumor location						
	One lobe	Both lobes				
Low	20	12	1			
High	54	18	1.8	0.005	695.559	0.846
Extra thyroidal invasion						
	absence	Presence				
Low	13	19	1			
High	56	12	6.821	2.66	17.487	0
Cervical lymph node metastasis						
	absence	Presence				
Low	15	17	1			
High	60	8	8.511	3.086	23.409	0
Angiolymphatic invasion						
	Presence	absence				
Low	19	13	1			
High	56	12	3.193	1.245	8.186	0.013

Discussion

In the present study, we observed that the PTC patients had significantly higher rates of -443CC genotypes than controls (P<0.001). The multivariate logistic regression analysis showed a significantly increased risk for PTC for the -443CC genotype compared with the -443TT genotype (adjusted OR= 4.312, 95%CI: 2.747 -6.987, adjusted P < 0.001). OPN was not expressed in normal thyroid tissues but was highly expressed in tumor sample from PTC patients. Also, we found that the high OPN expression were significantly more prevalent in -443CC carriers than CC carriers (P <0.001). Both the -443C>T polymorphisms and the tumor tissue OPN expression were closely associated with the cervical lymph node metastasis and angiolymphatic invasion of PTC. This study provides evidence to support the connection between the OPN genetic polymorphism and tissue expression with risk as well as the invasiveness of PTC.

Previous research has elucidated that OPN is up-regulated in a variety of cancers, such as breast, gastric, and colorectal cancers as well as in some highly metastatic cancer cell lines [12, 13]. High OPN levels in plasma and tissue were associated with shorten survival of patients with advanced cervical cancer [30]. Low OPN levels were significantly associated with a favorable prognosis with advanced non-small cell lung cancer [31], laryngeal and hypopharyngeal carcinomas[32], hepatocellular carcinoma[33], colorectal cancer[34, 35], upper urinary tract urothelial carcinoma [36], oral squamous cell carcinoma [37, 38], and endometrial cancer [39, 40]. OPN is classified as a Th1 cytokine/chemokine. One previous study reported the expression of Th1 chemokine (C-X-C motif) ligand 10 in anaplastic thyroid cancer, but not in the normal thyroid follicular cells [41] . In agreement with previous studies, we also found that high OPN expression in thyroid tissue of PTC patients while non-tumor thyroid tissue had no OPN expression. More interestingly, we found that the OPN expression levels were associated with the invasiveness of PTC. In addition, high OPN expression conferred higher rate of cervical lymph node metastasis and angiolymphatic invasion of PTC. Recent studies have reported that OPN plays important roles in cellular adhesion and migration, tissue repair, signal transduction and in the invasion and metastasis of several cancers at the molecular level [42, 43], suggesting that OPN expression could be used as a biomarker for PTC invasiveness.

The OPN encoding genes mapped on human chromosome 4q21-q25 and polymorphisms in the OPN gene promoter may affect its transcriptional activity [44]. More than fifty single nucleotide polymorphisms have been identified in the human OPN encoding gene in different populations, of which three single nucleotide polymorphisms on the promoter region of OPN gene, namely, -66T>G (rs28357094), -156G>GG (rs17524488), and -443C>T (rs11730582), were the most studied [44]. A previous study explored the possible role of −443T>C for OPN expression in melanoma cells. The authors found that the -443CC genotype had higher levels

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of OPN mRNA compared with other allelic variants and -443C>T variants might influence the OPN mRNA levels via binding of c-Myb transcription factor [45]. In oral squamous cell carcinoma (OSCC) patients, the -443 T/T genotype was found to be more prevalent in OSCC patients [25]. Our data showed that the only genotype of -443C>T were significantly related to the PTC risk, rather than the other two. More importantly, we observed that the high OPN expression was more frequent in samples from -443CC carriers than TT carriers. This is consistent with the result of a previous study, which explored the promoter activity of the -443 C>T polymorphism using a dual luciferase reporter assay. The author found that significantly higher luciferase activities were observed in the pGL3-C construct compared to the pGL3-T construct [26].

As far as PTC clinical features are concerned, -443CC carriage was significantly associated with higher risk for cervical lymph node metastasis and angiolymphatic invasion compared with -443 TT carriage. These data imply that the -443 C>T polymorphism might affect the PTC risk and invasiveness by influencing the OPN expression in tumor tissues. The -443CC of -443C>T polymorphisms was significantly associated with the angiolymphatic invasion and extra thyroidal invasion. This study suggests the -443C>T polymorphisms and tissue OPN expression may be used as molecular marker for the PTC incidence and invasion, including cervical lymph node metastasis and angiolymphatic invasion.

Most thyroid cancers have an excellent prognosis; both papillary and follicular thyroid cancers have about 85% to 90% cure rates, if detected early and treated appropriately. A deeper understanding of the genetic expression of OPN could lead to better treatment and prevention.

Some limitation in this study should be addressed. Firstly, this is a single center based study, which included Chinese patients from northern China, specifically Liaoning, Jilin and Heilongjiang provinces. The associations of OPN gene polymorphism with PTC need to be duplicated in other ethnic populations. Secondly, even though we found the association of OPN gene polymorphism and expression with PTC invasiveness, we did not have a follow-up study to investigate their prognostic role in PTC patients.

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