

RESEARCH ARTICLE

Overexpressed Osteopontin-c as a Potential Biomarker for Esophageal Squamous Cell Carcinoma

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Abstract

Background: The metastasis gene osteopontin (OPN) is subject to alternative splicing, which yields three messages, osteopontin-a, osteopontin-b and osteopontin-c. Osteopontin-c is selectively expressed in invasive, but not in noninvasive tumors. In the present study, we examined the expression of OPN-c in esophageal squamous cell carcinomas (ESCCs) and assessed its value as a diagnostic biomarker. **Methods:** OPN-c expression was assessed by immunohistochemistry in 63 ESCC samples and correlated with clinicopathologic factors. Expression was also examined in peripheral blood mononuclear cells (PBMCs) from 120 ESCC patients and 30 healthy subjects. The role of OPN-c mRNA as a tumor marker was investigated by receiver operating characteristic curve (ROC) analysis. **Results:** Immunohistochemistry showed that OPN-c was expressed in 30 of 63 cancer lesions (48%) and significantly associated with pathological T stage ($P=0.038$) and overall stage ($P=0.023$). Real time PCR showed that OPN-c mRNA was expressed at higher levels in the PBMCs of ESCC patients than in those of healthy subjects ($P<0.0001$) with a sensitivity as an ESCC biomarker of 86.7%. **Conclusion:** Our findings suggest that expression of OPN-c is significantly elevated in ESCCs and this upregulation could be a potential diagnostic marker.

Keywords: OPN-c - esophageal squamous cell carcinoma - biomarker - immunohistochemistry

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Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the least studied and most lethal cancers worldwide (Vizcaino et al., 2002; Lin et al., 2012; Wang et al., 2013; Ma et al., 2013), with a 5-year survival rate of less than 10% (Hofstetter et al., 2002; Lee et al., 2005). Notwithstanding primary prevention, screening, surgical treatment, and radiotherapy, the long-term survival rate of patients with ESCC has remained substantially unchanged in the last two decades. The main reason for the poor outcome of ESCC patients is the aggressive behavior of this malignancy, characterized by infiltrative and systemic spread, which results in frequent local recurrence and regional or distant lymph node metastasis. Therefore, identifying and targeting genes associated with the progression of ESCC is necessary to improve the survival of patients.

Osteopontin (OPN), which is also known as bone sialoprotein I (BSP-1) and secreted phosphoprotein 1 (SPP1), is an integrin-binding glycoprotein that is produced by cells of the immune system, epithelial tissue, smooth muscle cells, osteoblasts, and tumor cells. OPN has

many domains that support its various functions, including arginine-glycine-aspartic acid (RGD), binding sites for other cell surface receptors, as well as calcium- and heparin-binding regions. OPN also serves as a substrate for thrombin and matrix metalloproteinases (MMP2, MMP3, MMP7, MMP9 and MMP12). It supports migration and protects cells from programmed cell death by binding to integrin receptors, including $\alpha v\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 5$ (Senger et al., 1996; Sodek et al., 2000; Barry et al., 2000; Furger et al., 2003) or CD44 (Katagiri et al., 1999; Weber et al., 1996). OPN is overexpressed in many human tumors including colon, ovarian, breast, liver, prostate, and lung carcinomas (Chambers et al., 1996; Thalmann et al., 1999; Tuck and Chambers 2001; Agrawal et al., 2002; Ye et al., 2003; Schorge et al., 2004). Elevated expression of OPN is often correlated with malignancy and has been shown to increase the tumorigenic and metastatic phenotype of cancer cells. Transfection with antisense oligonucleotides yields a population with a reduced malignant potential (Gardner et al., 1994).

There are three splice variants for OPN, OPN-a, OPN-b and OPN-c. OPN-b lacks exon 5 and OPN-c lacks exon 4. OPN-a is expressed in both cancer and normal

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subjects, and OPN-b is difficult to detect because of its low expression level (Mirza et al., 2008). OPN-c is expressed at high levels in breast cancer tissues, whereas it is not detected in surrounding normal tissue. Because OPN was up-regulated in the serum and cancerous tissue of ESCC patients, and overexpressed osteopontin was associated with lymph node metastasis and poor survival of ESCC patients, its potential as a target for ESCC therapy has been explored (Ito et al., 2006; Mirza et al., 2008). Despite extensive research on OPN in cancer, the expression level and role of OPN-c in ESCC remains unknown. In the present study, we examined the expression of OPN-c in the tissue and peripheral blood mononuclear cells (PBMCs) of ESCC patients and evaluated its clinical application for the early diagnosis of ESCC.

Materials and Methods

Patients and samples

A total of 63 patients with esophageal cancer who were admitted to Jiangsu cancer hospital were enrolled in this study. All patients had histological verification of ESCC, and each patient was classified according to the pathological tumor-node-metastasis (pTNM) system. The age of patients ranged between 39 and 79 years, with a mean of 59.7. Patients who received preoperative chemotherapy or radiotherapy were excluded from this study. Tissue samples were obtained from the department of pathology. Each sample had been fixed in formalin, routinely processed, and embedded in paraffin. A volume of 2 mL of pre-operation venous blood was drawn into EDTA-K2 anticoagulant tubes from 120 patients with ESCC. Venous blood was also collected from 15 post-operation ESCC patients out of the 120 ESCC patients. PBMCs were separated using Ficoll-Hypaque. Informed consent was obtained from all the patients. 30 healthy individuals served as controls.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue blocks were cut into 6 μ m thick sections, which were baked at 50–60°C for at least 2 h, deparaffinized with xylene and rehydrated through a graded alcohol series. All specimens were subjected to heat-induced antigen retrieval in 10 mM sodium citrate buffer (pH 6.0). The slides were incubated with Tris-buffered saline (TBS) supplemented with 5% goat serum to block nonspecific binding. Sections were incubated with the primary antibody, anti-OPN-c (1:160 dilution, Gallus Immunotech, Canada) for 2 h. After rinsing with phosphate buffered saline (PBS) for 15 min, sections were incubated with secondary antibody goat anti-chicken IgY (1:200 dilution, KPL, USA) for 30 min and washed again with PBS for 10 min. Substrate DAB was added and followed by hematoxylin counter-staining. Breast cancer tissue was included as a positive control. The frequency of OPN-c positive cells was scored on the basis of the percentage of positive cells as 0% = negative; 1–25% = +1; 26–50% = +2; and >51% = +3. The intensity of OPN-c expression was scored as weak = 1, moderate = 2 and strong = 3. The average OPN-c expression of each section was calculated as intensity multiplied by

frequency and classified as low (≤ 2) or high (> 2). All of the sections were scored twice to confirm the reproducibility of the results.

RT-PCR

Total RNA was extracted with DNase treatment from PBMCs using the Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. First-strand cDNA was synthesized using M-MuLV Reverse Transcriptase (Fermentas, EU). Real-time PCR reactions were then carried out in a total of 20 μ L of reaction mixture, which was composed of 7 μ L of cDNA, 10 μ L of 2 \times SYBR Green PCR Master Mix (TaKaRa), 0.4 μ L of each 10 μ mol/L forward and reverse primers, and 2.2 μ L of H₂O. Primers for OPN-c used in the experiment were 5'-TGAGGAAAAGCAGAATGCTG-3'

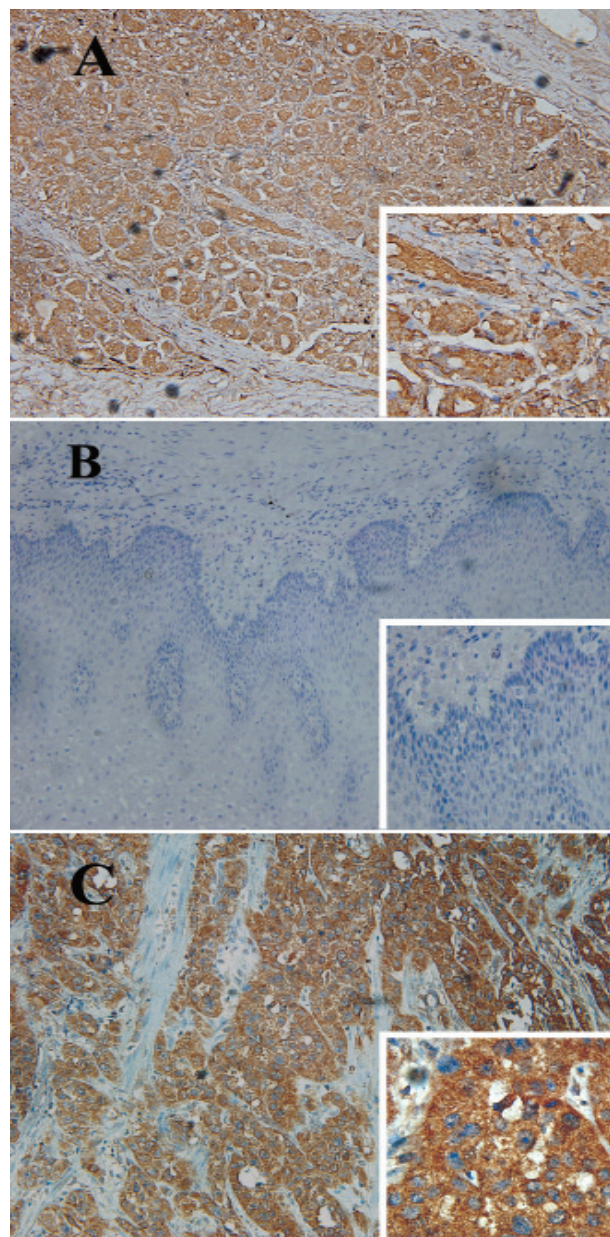


Figure 1. Expression of OPN-c was Measured by Immunohistochemistry. (A) control of positive staining of OPN-c on breast cancer ($\times 200$); lower right corner ($\times 400$); (B) Expression of OPN-c is negative in adjacent non-cancerous tissue; lower right corner ($\times 400$); (C) Positive staining of OPN-c in ESCC cancerous tissue ($\times 200$); lower right corner ($\times 400$)

Table 1. Relationship Between OPN-c Expression in ESCC with Clinicopathologic Factors

Case	OPN			χ^2	P
	Total (n=63)	High n=30(47.6%)	Low n=33(52.4%)		
Age					
≤60	26	13(43)	13(39)	0.101	0.751
> 60	37	17(57)	20(61)		
Gender					
Male	56	26(87)	30(91)	0.286	0.593
Female	7	4(13)	3(9)		
Smoke					
Yes	24	10(33)	14(42)	0.551	0.458
No	39	20(67)	19(58)		
Alcohol					
Yes	24	11(37)	13(40)	0.05	0.824
No	39	19(63)	20(60)		
Histology					
Well	3	1(3)	2(6)	0.268	0.875
Moderate	52	25(83)	27(82)		
Poorly	8	4(14)	4(12)		
Tumor diameter					
<4 cm	53	23(77)	30(91)	2.387	0.122
≥4 cm	10	7(23)	3(9)		
pT					
pT1	5	1(3)	4(12)	8.426	0.038
pT2	17	4(13)	13(39)		
pT3	33	20(67)	13(39)		
pT4	8	5(17)	3(10)		
Stage					
I	5	1(3)	4(12)	7.51	0.023
II	23	7(23)	16(48)		
III	35	22(74)	13(40)		
Lymph node metastasis					
Yes	16	6(20)	10(30)	0.88	0.348
No	47	24(80)	23(70)		

(forward), 5'-GTCAATGGAGTCCTGGCTGT-3' (reverse). The amplification of beta-actin with primers 5'-GGCGGCACCACCATGTACCCT-3' (forward) and 5'-AGGGGCCGGACTCGTCATACT-3' (reverse) served as a control. The PCR yielded products of 155 bp for OPN-c. PCR reactions were carried out in Roche lightcycler 1.5 as follows: 30 s at 95°C, followed by 45 cycles of 15 s at 95°C, 15 s at 55°C and 45 s at 72°C. Melting curves for each PCR reaction were generated to ensure the purity of the amplification product. Products were analyzed using agarose gels. All PCR amplifications lead to a single and specific product. The expression of OPN-c mRNA in PBMCs compared to beta-actin mRNA was determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Statistical analysis

The analyses of data were performed using the software package SPSS16.0 for Windows. The Mann-Whitney test was used to examine significant differences of OPN-c mRNA expression between healthy and cancer groups. The χ^2 test was utilized to evaluate the correlations between the expression of OPN-c and the clinicopathological factors. The level of critical significance was considered to be $P < 0.05$. Receiver operating characteristic curve was used to estimate the value of OPN-c mRNA in PBMCs as a tumor marker.

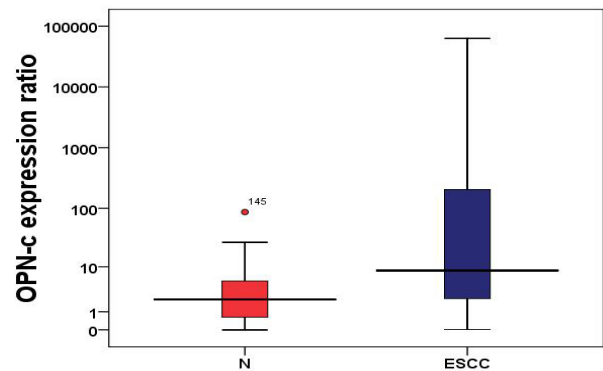


Figure 2. Expression Ratio of OPN-c mRNA on PBMC in N (PBMC from normal group) and ESCC (PBMC from ESCC group)

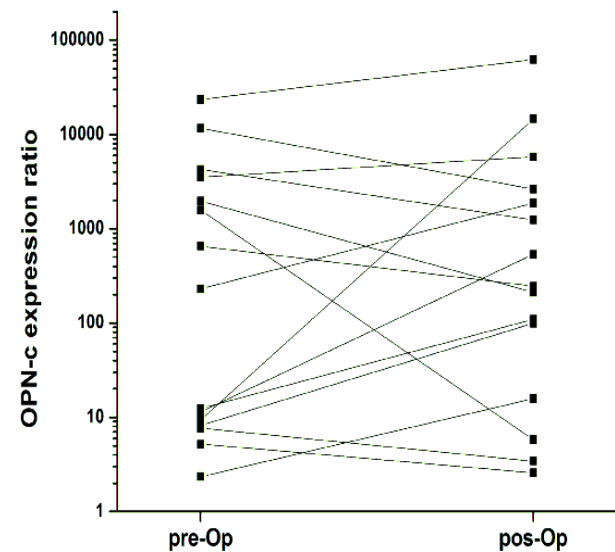


Figure 3. Expression Ratio of OPN-c in Patients (n=15) with ESCC Before (pre-Op) and 7 Days after (7 day post-Op) Surgical Removal of the Tumor

Results

Immunohistochemical detection of OPN-c in ESCC

The expression and localization of OPN-c were detected by immunohistochemistry in 63 ESCC samples, of which 30 (48%) stained positive for OPN-c and 33 stained negative. Representative results of OPN-c staining were shown in Figure 1. The relationship between OPN-c expression and clinicopathologic features of ESCC patients was shown in Table 1. OPN-c expression was significantly associated with pathological T stage ($P=0.038$) and overall stage ($P=0.023$). However, no significant correlation was observed between OPN-c expression and histology ($P=0.875$) or lymph node metastasis ($P=0.348$).

OPN-c mRNA expression in peripheral blood mononuclear cells

The expression of OPN-c mRNA was examined in PBMCs from ESCC patients and healthy controls by real time PCR. OPN-c mRNA levels were higher in PBMCs of ESCC patients (Median=8.52, 95% confidence interval (CI) = 5.34–17.55) than in those of healthy controls

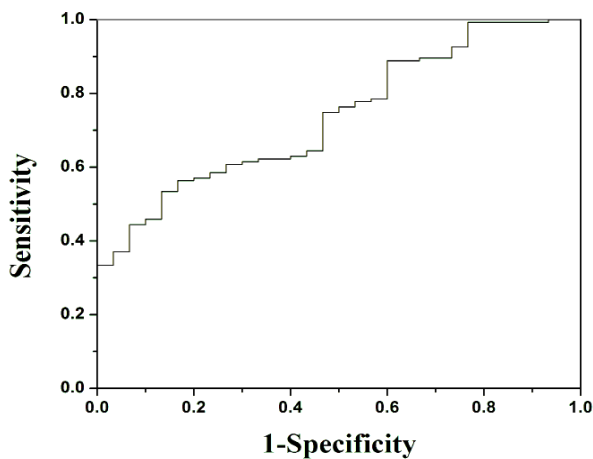


Figure 4. Receiver Operating Characteristic Curve of OPN-c mRNA on PBMC

(Median=2.19, 95% CI = 0.85–4.94) ($P < 0.0001$, Figure 2). OPN-c mRNA was undetectable in the PBMCs of 8 out of 30 normal specimens. Assessment of the preoperative and postoperative OPN-c mRNA levels in the PBMCs of 15 patients with ESCC showed no differences between preoperative and postoperative OPN-c expression ($P = 0.76$, Figure 3).

OPN-c mRNA expression in PBMCs as a marker of ESCC

The significance of OPN-c mRNA expression in PBMCs as an ESCC marker was evaluated. The sensitivity of OPN-c mRNA expression in PBMCs as a marker of ESCC was 86.7%. The area under the ROC curve (AUC) was 0.738 (Figure 4). Positive likelihood ratio was 1.86 and negative likelihood ratio was 0.25.

Discussion

OPN is a glycoposphoprotein that is overexpressed in various cancers and is involved in tumorigenesis. Identifying the clinical characteristics associated with the malignant behavior of ESCC is critical to improve the outcome of patients. In the present study, we showed positive staining for OPN-c by immunohistochemistry in 48% of ESCC tissues, which was consistent with previous immunohistochemical studies of OPN in patients with ESCC (Kita et al., 2006). OPN-c expression was correlated with certain clinicopathologic characteristics, such as tumor invasion and tumor stage. Alternative splicing prevents OPN from aggregating, thus increasing the amount of soluble OPN available for binding receptors and promoting the anchorage independence of ESCC cells. OPN-c lacking exon 4 was compared with the standard form of OPN and shown to strongly support anchorage independent growth (He et al., 2006).

Because the clinical symptoms of ESCC are usually detected at the late stages of the disease, there is a need for better early detection methods. Despite the identification and characterization of a significant number of tumor markers for ESCC, a useful screening marker for ESCC has not been clearly established. Here, we used real time PCR to investigate the expression of OPN-c in PBMCs and showed that the levels of OPN-c mRNA were higher

in the PBMCs of ESCC patients than in those of healthy controls. Furthermore, because PBMCs are relatively easy to obtain, we assessed the value of OPN-c mRNA levels in PBMCs as a tumor marker. Our results showed that OPN-c level could served as a promising sensitivity as a marker for patients with ESCC. Because OPN-c is expressed at high levels in association with several other malignancies, it cannot be considered as a specific tumor marker for ESCC. However, the present study was a retrospective analysis and additional prospective clinical trials are necessary to confirm our results.

In conclusion, OPN-c was expressed at high levels in ESCC tissues and was closely related to invasion and stage of cancer. OPN-c mRNA expression in PBMCs could be a potential tumor marker for the early detection of ESCC.

Acknowledgements

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