Original Article
Functional characterization of OPN in human laryngeal squamous cell carcinoma and its xenograft model in nude mice

Jianqiu Chen[1], Qi Zhou[2], Chunsheng Zhu[1], Minhui Zhu[3], Yongsheng Tian[4], Guojun Li[5], Xiaofeng Tao[6], Hongliang Zheng[3]

[1]Department of Otolaryngology Head and Neck Surgery, General Hospital of Jinan Military Region, Jinan 250031, Shandong Province, PR China; [2]Department of Tumor Biological Treatment, The Third Affiliated Hospital, Soochow University, Changzhou 213003, Jiangsu Province, China; [3]Department of Otolaryngology Head and Neck Surgery, Changhai Hospital of The Second Military Medical University, Shanghai 200433, PR China; [4]Department of Otolaryngology Head and Neck Surgery, Aerospace Center Hospital of Beijing university, Beijing 100049, PR China; [5]Department of Head and Neck Surgery, U.T. M.D. Anderson Cancer Center, Houston, TX 77030, USA; [6]Department of Radiology, Shanghai Ninth People's Hospital Affiliated Shanghai Jiaotong University School of Medicine, China

Abstract: Background: Osteopontin (OPN) is involved in promotion of cancer cells by regulating various facets of tumor progression such as cell proliferation, angiogenesis and metastasis. To understand the role of OPN in laryngeal squamous cell carcinoma (LSCC), we thus explored the biological function of OPN in LSCC after silencing OPN expression by RNA interference (RNAi). Method: The OPN expression in tumor tissues of LSCC was determined immunohistochemically in both LSCC and adjacent normal tissues. Lentivirus vector with RNAi small hairpin gene sequence of OPN (named LV-shOPN) was transfected into Hep-2 cells and transplanted into BALB/c-nu mice. After siRNA transfection, the viability of Hep-2 cells was examined by MTS, OPN expression was detected by Western blotting, and tumor angiogenesis was assessed by microvessel densities (MVD). Results: The difference of positive rate of OPN in 72 cases LSCC (54 cases, 75.0%) and adjacent normal tissues (15 cases, 20.8%) was statistically significant (P<0.001) and the OPN expression was also significantly correlated with tumor stage, grade and the presence of lymph node. Hep-2 cells infected with LV-shOPN significantly decreased OPN expression, in comparison to cells with LV-shNon transfection (as the control) (P<0.05). The constructed LV-shOPN effectively inhibited the viability of Hep-2 cell and growth of xenograft tumors in nude mice (all P<0.050). The expression of OPN and MVD was significantly decreased in xenograft tumors (all P<0.05). Conclusion: RNAi silencing of OPN expression can significantly inhibit tumor growth and angiogenesis of Hep-2 cells, and OPN may be considered as one of gene targeting therapy for LSCC.

Keywords: Osteopontin, RNA interference, laryngeal neoplasms, nude mice, laryngeal squamous cell carcinoma

Introduction

Laryngeal squamous cell carcinoma (LSCC) is one of the most common types of head and neck squamous cell carcinoma. LSCC accounts for 1-2% of all malignancies diagnosed worldwide [1]. The malignant biological behavior, such as tumor recurrence, metastasis, multidrug resistance, and resistance to radiochemotherapy, leads to worse prognosis and poor life quality of these patients. Metastasis and recurrence of LSCC are the main primary causes of

failure for treatment and subsequent death in patients with LSCC despite advances in diagnostic and therapeutic approaches. Thus, there is a critical need not only to delineate the molecular underpinning of human LSCC, but also to identify molecular targets for effective therapeutic interventions.

Osteopontin (OPN), also known as secreted phosphoprotein 1 (SPP1), is involved in promotion of cancer cells by regulating various facets of tumor progression such as cell proliferation,
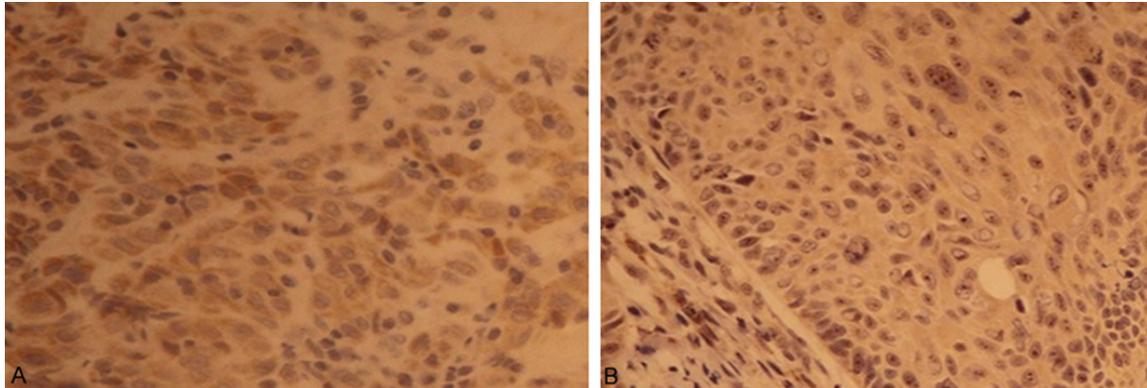
Figure 1. OPN expression in LSCC tissues (A) and adjacent nontumor tissues (B), immunohistochemical staining (×400).

Table 1. Associations between OPN expression and clinicopathological characteristics of LSCC

Variables	N	OPN expression		P values
		High	Low	
Age (yr)				0.466
<60	23	16	7	
≥60	49	38	11	
Gender				0.442
Male	67	51	16	
Female	5	3	2	
Tobacco				0.612
Ever	52	40	12	
Never	20	14	6	
Alcohol				0.475
Ever	47	34	13	
Never	25	20	5	
Tumor sites				0.851
Supraglottic	28	20	8	
Glottic	40	31	9	
Infraglottic	4	3	1	
Overall stage				0.013*
I-II	30	18	12	
III-IV	42	36	6	
Tumor differentiation				0.014*
Well	34	21	13	
Moderate/poor	38	33	5	
Lymph node metastasis				0.001*
Positive	33	31	2	
Negative	39	23	16	
Treatment				0.492
Surgery only	31	22	9	
Combined	41	32	9	

*$P < 0.05$.

angiogenesis and metastasis. OPN is a highly acidic calcium-binding glycosylated phosphoprotein [2-4] which functions as a cell attachment protein and cytokine that signals through two cell adhesion molecules, $\alpha v\beta 3$-integrin and CD44, to regulate cancer growth and metastasis [5]. Binding of OPN to these cell surface receptors stimulates cell adhesion, chemotaxis, proliferation, migration, and specific signaling functions [6, 7]. OPN may play multiple roles in promoting tumor progression, including inhibiting macrophage function and enhancing growth or survival of metastases [8]. Overexpression of OPN has been found in a variety of cancers, including breast cancer, lung cancer, colorectal cancer, stomach cancer, ovarian cancer, esophageal cancer and melanoma [9-11]. Moreover, OPN is present in elevated levels in some patients with metastatic cancers [12-14]. OPN expression was significantly correlated with differentiation and lymphatic metastasis of the LSCC. Elevated OPN level was significantly associated with poor survival [15]. Therefore, suppression of the action of OPN may confer significant therapeutic activity, for which several strategies have been identified [16, 17]. Although OPN is overexpressed in many human malignancies, the biological function of OPN in LSCC has not been fully understood. Thus, further studies of the role of OPN in carcinogenesis and its clinicopathologic characteristics in LSCC are needed. In this study, we investigated OPN expression in LSCC specimens and its correlation with clinicopathologic characteristics. Furthermore, we also used small hairpin RNA (shRNA), a powerful therapeutic potential tool by silencing oncogenes, to detect the potential function by RNA
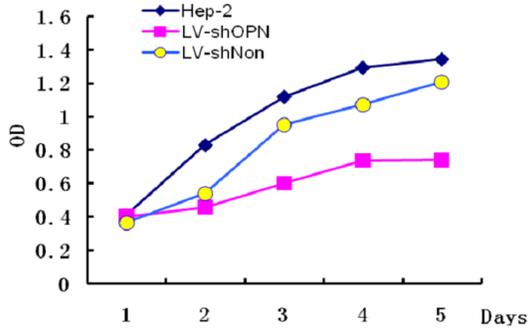
Figure 2. Inhibition of LV-shOPN in Viability of Hep-2 cells.
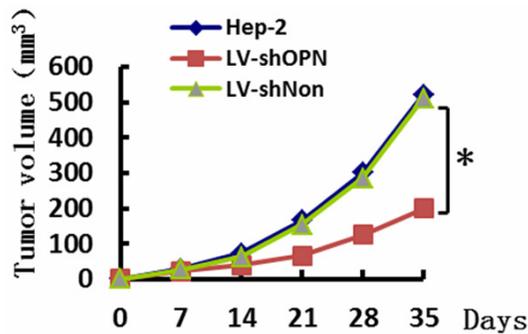
Figure 3. The curve of tumor growth of nude mice with LV-shOPN in LSCC.

interference (RNAi) silencing OPN expression in human LSCC xenograft in nude mice.

Material and methods

Patients and specimens

All 72 tumor samples were obtained from patients who had undergone surgery and were diagnosed with primary LSCC in General Hospital of Jinan Military Region from January 2003 to December 2006. The adjacent nontumor tissues were about 1 cm from primary carcinoma. Histopathological diagnoses were made according to the pathological classification system of the World Health Organization [18], and the tumor was staged following the tumor-node-metastasis classification of the International Union Against Cancer (UICC 2002). The clinicopathological information of patients was available, including gender, age, tumor site, tobacco, alcohol, tumor stage, histological grade, and metastasis. All patients didn't receive radiotherapy, chemotherapy and immunotherapy before the surgery.

Western blot analysis

Cells were washed in phosphate buffered saline, and lysed directly using RIPA buffer. Proteins at the same amount were separated by 12% SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. After probing with antibodies, the signals were visualized by supersignal enhanced chemiluminescence reagent (Pierce, Rockford, IL). The antibodies used were anti-osteopontin (R&D Systems, Minneapolis, MN) and anti-glyceraldehyde-3-phosphate dehydrogenase (KangChen Bio-tech, Shanghai, P. R. China). Gel bands were then developed with ECL Western blotting detection reagents (Amersham, USA).

Immunohistochemical staining

Immunohistochemical staining was performed on thin sections (~ 4 µm) of paraffin-embedded archival poly-L-lysinecoated glass slides. The samples were dewaxed in 100% xylene and rehydrated in descending ethanol series and water according to standard protocols. Heat-induced antigen retrieval was performed in 0.001 mol/L EDTA buffer for two minute at 100°C. The slides were fixed and the endogenous peroxidase activity was quenched by incubation in methanol with 3% hydrogen peroxide for 10 minutes. The slides were then washed with phosphate-buffered saline. Nonspecific binding was blocked by incubation with 2% bovine serum in Tris-buffered saline for 30 minutes at room temperature. The slides were first incubated with the primary monoclonal mouse-anti-human antibody against OPN (Zhongshan Technology Co., Beijing, China) overnight at 4°C, and the slides were then washed 3 times in phosphate-buffered saline containing 3%, 2%, and 1% of normal human serum. Biotinylated anti-mouse-IgG (Zhongshan Technology Co., Beijing, China) avidin-biotin complex was applied as a secondary antibody for 30min at room temperature, followed by the application of peroxidase-conjugated strepavidin for 30 min. The antibody binding was visualized with 3, 3-diaminobenzidine tetrahydrochloride before brief counterstaining with Mayer's hematoxylin. For monoclonal antibodies of mouse origin, negative controls were obtained using isotypic mouse immunoglobulin in the same dilution as the primary antibody of con-
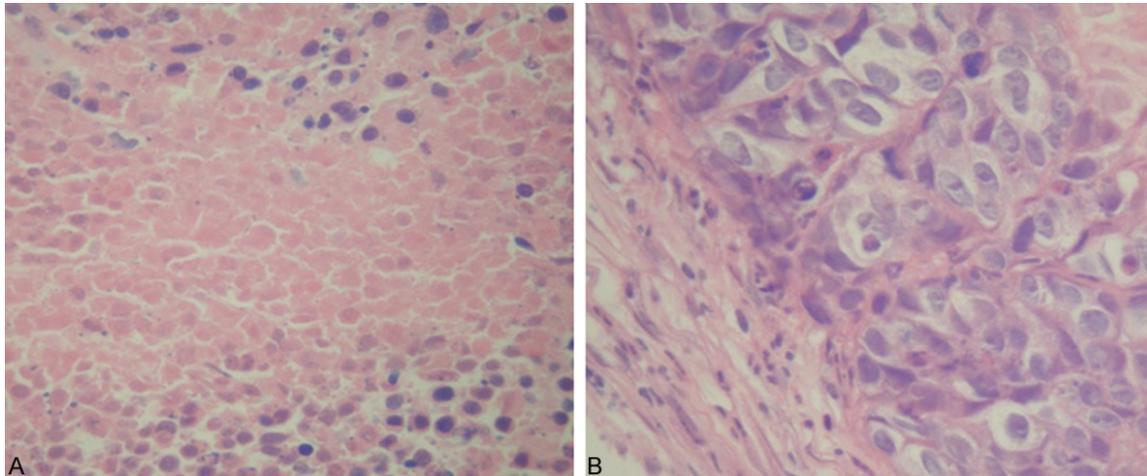
Figure 4. HE staining of xenotransplanted tumors (×400). A: LV-shOPN group. B: LV-shNon control group.
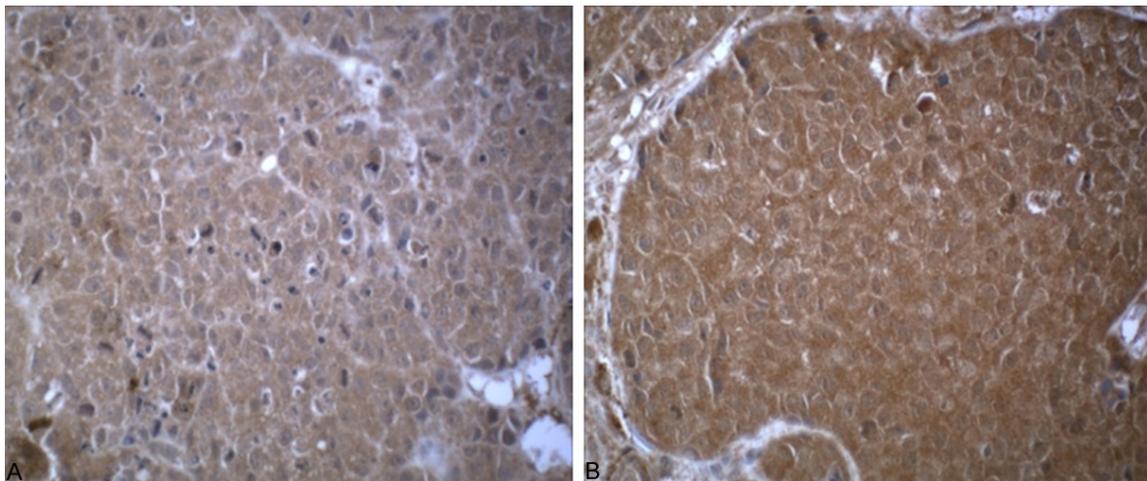
Figure 5. Expression of OPN in transfected LV-shOPN Hep-2 xenografts (×400). A: LV-shOPN group. B: LV-shNon control group

cern. All control experiments gave negative results.

Evaluation of immunostaining

Evaluation of OPN protein staining was independently performed by 2 experienced pathologists in a blinded fashion. Five representative microscopic areas at ×400 magnification were randomly selected for examination. Expression of OPN was determined by both the intensity of staining and the proportion of tumor cells that had an unequivocal positive reaction using a semi-quantitative and subjective grading system [19]. A proportion score was assigned, which represented the estimated proportion of positively stained tumor cells (0, none; 1, <10%; 2, ≥10% to <50%; 3, ≥50% to <80%; 4, ≥80% to <100%). An intensity score was assigned that represented the average intensity of the positive tumor cells (0, none; 1, weak; 2, intermediate; 3, strong). Multiplication of the intensity and the proportion scores gave rise to the ultimate immunohistochemical scores: a total score greater than or equal to 3 was taken to indicate a high expression and a sum score below 3 indicated a low expression.

Cell culture and lentiviral infection

The Hep-2 cells of human LSCC were provided by the Center Laboratory, Second Military
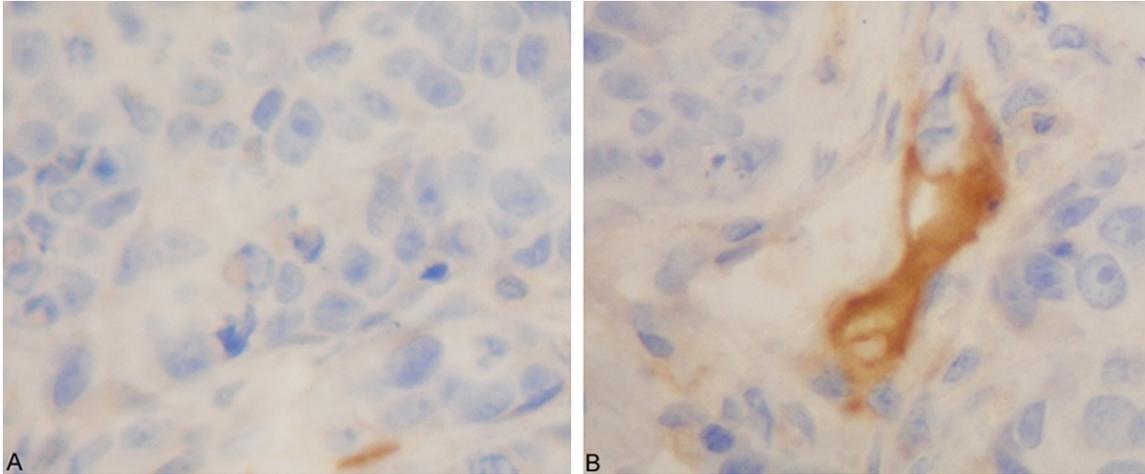
Figure 6. CD34 expression of of xenotransplanted tumors (×400). A: LV-shOPN group. B: LV-shNon control group.
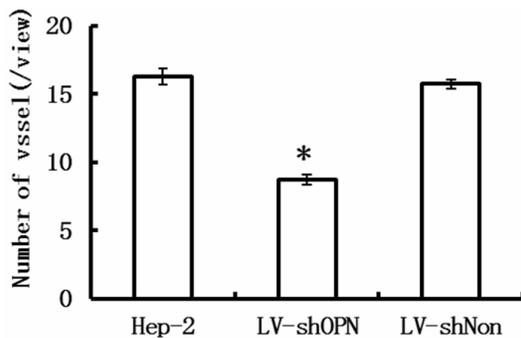
Figure 7. Numbers of MVD by CD34 expression: LV-shOPN and LV-shNon group (8.78±1.30 and 15.72±1.50, respectively).

Medical University. Cells were cultured in DMEM medium containing 10% fetal bovine serum (Gibco) and incubated in a humidified (37°C, 5% CO_2) incubator. Small hairpin RNA (shRNA) of human OPN lentivirus gene transfer vector (named LV-shOPN) encoding green fluorescent protein (GFP) sequence was constructed and provided by Dr. Zhu Minghui. Lentivirus vector carried GFP but without OPN was named LV-shNon as a control group.

Hep-2 cells were plated in 24-well plates (2×10^4 cells/well) overnight. The lentiviruses were diluted in 0.2 mL (10^8 TU/mL) complete medium containing polybrene (8 mg/mL) and added to the cells for incubation for 1 h at 37°C, followed by incubation in 0.3 mL of freshly prepared polybrene-DMEM for another 24 h, then the medium was replaced with fresh DMEM and the cells were cultured for another 48 h.

Cell viability assays

Cell viability was assessed by MTS assay (Promega, Madison, WI) in 96-well plates (5000 cells/well) following the instructions of the manufacturer. Each experiment was done in triplicate and repeated three times.

In vivo tumor xenograft model in nude mice

A total of 18 BALB/c-nude mice, 5-6 weeks old and 20 g in weight, were provided by Vital River Laboratory (Laboratory Animal Technology Co., Beijing, China), were bred in an aseptic condition, and kept at a constant humidity and temperature (25-28°C) in the pathogen-free animal facility at least one week before use. For the tumorigenic assay, all the 18 mice (6 mice/group) underwent subcutaneous injection of 200 μL (1×10^7) viable cells suspension with LV-shOPN or LV-shNon in the dorsal scapula region. The size of tumors was blindly measured once a week with calipers, and the volume of tumor was determined using the simplified formula of a rotational ellipsoid ($L \times W^2 \times 0.5$), where L is the largest dimension and W is the perpendicular diameter. Upon termination of the experiment, mice were sacrificed and individual tumors were weighted and collected for further analysis.

Histomorphologic assessment of tumor xenograft and immunohistochemical staining

Resected tumors were fixed in 4% Polyoxymethylene, processed in paraffin, sectioned

at 4 μm, and subjected to a hematoxylin and eosin staining protocol. The stained tissues were observed under a light microscope. Immunohistochemical staining of tumor xenografts was performed as described above.

Analysis of tumor vascular density

Tumor microvessel density (MVD) was quantified using sections immunostained for CD34 (Santa Cruz, CA, USA) by two independent investigators in a blinded manner as described previously [20]. The areas with the greatest density of CD34-positive endothelial cells were designated as "hot spots" The whole section was scanned at low power (×40) to identify the best fields for counting. Counting was performed on five separate fields within a hot spot at ×200 magnification. Each stained endothelial cell or cell cluster was counted as one microvessel. If two or more CD34-positive foci appeared to belong to a single continuous vessel, then this was counted as one microvessel. The mean vessel count from these fields was used for MVD scoring.

The investigation was approved by the Ethics Committee of General Hospital of Jinan Military Region. Written informed consent was obtained from all patients. Experiments were approved by the local authorities and the Hospital Animal Care and Use Committee (Jinan, Shandong China).

Statistical analysis

The difference in OPN expression between LSCC and adjacent nontumor tissue was performed using the Student's *t*-test and one-way analysis of variance for multiple comparisons. Chi-square test was used to analyze the associations between OPN expression and clinicopathologic parameters. The SPSS software (for Windows, version 19.0) was used for statistical calculations. A *P* value of less than 0.05 was considered statistically significant.

Results

Expression of OPN in LSCC

The age of 72 LSCC patients, including 67 men and 5 women, ranged from 33 to 76 years old, with a mean of 60.5 years. Of 72 patients, OPN positive staining was detected in 54 (75.0%)

and 15 (20.8%) in LSCC and adjacent nontumor tissues, respectively. The staining of OPN was observed in cytoplasm and cell membrane, but no nuclear staining was found in all tissues studied. The brown or yellow-brown grana or bolus was shown in cytoplasm of positive staining cells. OPN expression was higher in LSCC than in adjacent normal tissue (P<0.001) (**Figure 1A**, **1B**).

Correlation of OPN expression with selected clinicopathologic characteristics of LSCC

The clinicopathologic characteristics of 72 patients with LSCC are listed in **Table 1**. A significant correlation was found between the OPN expression and overall stage, tumor differentiation, and lymph node metastasis (P = 0.013; P = 0.014 and P = 0.001, respectively). However, such significant correlation was not observed for other variables including age, gender, smoking, alcohol use, tumor sites, and treatment (all P>0.05).

LV-shOPN reduced OPN expression in Hep-2 cells

The OPN expression was further analyzed by western blotting in Hep-2 cells. Hep-2 cells infected with LV-shOPN significantly decreased OPN expression, in comparison to cells with LV-shNon transfection as control (P<0.05). These results clearly indicated that the constructed LV-shOPN might effectively infect Hep-2 cells and significantly reduced OPN expression.

Decreased viability of Hep-2 cells with LV-shOPN transfection

As shown in **Figure 2**, after LV-shOPN transfection, the viability of Hep-2 cells was decreased in comparison with cells without and with LV-shNon transfection (P<0.05). Although the viability was borderline significantly different between the cells with LV-shNon transfection and the cells with blank control (Hep-2 cells only) (P = 0.051), the time-effect curves indicated that LV-shOPN transfection could decrease the viability of Hep-2 cells *in vitro*.

LV-shOPN suppressed tumor growth in vivo

The effect of OPN shRNAs on tumor growth was evaluated by using cancer xenograft volume

changes. Tumor volume of each group was scored every 7 days. Data was represented as means ± SD. LV-shOPN significantly inhibited tumor growth as shown in **Figure 3**. Tumors from mice with LV-shOPN were significantly smaller in volume, compared with those with LV-shNon or blank control group (P<0.05).

As shown in **Figure 4**, the HE staining of tumor xenografts showed that all the tumor tissues were squamous cell carcinoma. The tumor cells from the group with LV-shNon cells showed a vivid staining, rose pink cytoplasm, and dark purple nuclei in tumors, while the tumors originating from LV-shOPN-expressing cells showed a sparse structure, anomalous shapes of cells, fragmented or even disappeared cell bodies and lightly stained or condensed nuclei, reminiscent of a necrosis morphology (**Figure 4**). It was likely that interference of OPN into Hep-2 may inhibit tumor growth.

Inhibition of LV-shOPN in OPN expression in tumor xenograft

The significantly reduced expression of OPN by immunohistochemical staining was observed by down-regulation of OPN in Hep-2 xenografts after LV-shOPN transfection as compared with control tumors which had high expression of OPN (**Figure 5**). The expression of OPN was statistically different between the two groups (P<0.05).

Inhibition of LV-shOPN in tumor angiogenesis in vivo

Tumor-associated neovascularization, as indicated by MVD, was determined by CD34 expression in tumor sections derived from xenografts. As shown in **Figures 6** and **7**, a significant decrease in tumor MVD was observed by down-regulation of OPN in Hep-2 xenografts as compared with control tumors (P<0.05). It is likely that OPN might be involved in regulation of tumor angiogenesis.

Discussion

LSCC is one of the most common malignancies in head and neck. Despite modern detection and treatment strategies, the overall survival for this disease has improved only marginally over the past decade. Tumor recurrence/metastasis and second primary malignancy are the two major causes of unfavorable prognosis

[21, 22]. A tumor marker for early detection before the clinical manifestation of this cancer would be essential to better treatment and survival [21, 22]. Recently, it was reported that OPN may be a candidate of such markers for early detection of head and neck cancers [23]. In LSCC, the OPN expression is positively correlated with degree of dysplasia but negatively with survival [24]. In plasma and tumor tissue samples, the elevated OPN expression was also significantly associated with metastasis and survival of LSCC [15]. These findings may support that OPN may serve as one of useful indicators of tumorigenesis and prognosis for LSCC.

RNAi is known as a post-transcriptional gene silencing (PTGS) machinery in which double-stranded RNA (dsRNA), in tandem with protein complexes, catalyzes the degradation of complementary mRNA targets [25, 26]. Methods for applying RNAi to the treatment of hereditary diseases, various neurodegenerative diseases, and cancers as an antiviral therapy agent are currently under development. Small hairpin RNAs (shRNAs), the precursors of siRNAs, contain the sequence of the siRNA guide strand (21-29 bp long), followed by a loop consisting of approximately 9 nucleotides, and a sequence that is complementary to the siRNA guide strand. The use of this structure enables to achieve long-term suppression of gene expression [27]. Lentiviral vectors are optimal tools for the delivery of shRNAs into dividing and non-dividing cells, making their application promising for the treatment of malignancy tumors [28]. In the present study, we have also found that the OPN expression in LSCC tissues was significantly higher than that in adjacent normal tissues. The expression of OPN was significantly correlated with tumor stage, lymph node metastasis and poor differentiation, but such similar correlations were not observed for age, gender, smoking, alcohol use, tumor sites and treatment. Because of the increased expression of OPN in many other tumor tissues [9-11, 14], it is suggested that OPN may be a potential marker for tumor progression and may play important roles in invasion and metastasis of LSCC. It is likely that OPN may be a potentially therapeutic target for LSCC therapy.

In this study of LSCC, we used the lentivirus vector system to deliver a specially designed shRNA for human OPN gene into LSCC cell line,

Hep-2, to silence the expression of OPN and investigated the effect of decreased OPN on viability and growth of LSCC. We found that *in vitro* the Hep-2 cells with transfected LV-shOPN inhibited the growth and *in vivo* xenograft tumor volumes in mice bearing transfected LV-shOPN Hep-2 cells significantly reduced. Moreover, LV-shOPN led to cells anomalous shapes, even necrosis and significantly reduced the expression of OPN in xenograft tumor tissues. Thus, it was likely that OPN knock down by shRNA in LSCC might inhibit tumor growth *in vivo*, possibly by promoting necrosis of tumor regression by shRNA merely targeted to OPN.

Angiogenesis is a prerequisite for advanced tumor growth and is logically believed to be an important factor in tumor growth and metastasis [29]. The degree of angiogenesis is associated with tumor aggressiveness and clinical outcome [30, 31]. CD34 is a potent stimulator of angiogenesis and plays an important role in tumor growth and progression [32]. The assessment of MVD, detected by antibody to CD34, is frequently used to quantify angiogenesis in archival tissue [33]. Our results clearly showed a decreased expression of MVD by effectiveness of LV-shOPN against LSCC Hep-2 cells in tumor xenografts, suggesting that down-regulation of OPN signaling may inhibit tumor angiogenesis and reduce tumor invasion *in vivo*. Therefore, both *in vitro* and *in vivo*, silencing of OPN by shRNA could be directly linked to the inhibition of tumor growth and invasion.

Taken together, we conclude that delivery of shOPN using lentivirus may have potential for targeted gene therapy of human LSCC. RNA interference holds out great promise in the treatment of LSCC. Further larger studies and molecular mechanisms underlying roles of OPN in LSCC are needed.

Acknowledgements

Disclosure of conflict of interest

None.

Abbreviations

RNAi, RNA interference; OPN, osteopontin; LSCC, laryngeal squamous cell carcinoma; LV-shOPN, lentivirus vector with RNAi small hairpin gene sequence of OPN; MVD, microvessel density.

Address correspondence to: Xiaofeng Tao, Department of Radiology, Shanghai Ninth People's Hospital Affiliated Shanghai JIaoTong University School of Medicine, China. Tel: 02123271699-5335; Fax: 86-21-6316856; E-mail: cjr.taoxiaofeng@vip.163.com; Hongliang Zheng, Department of Otorhinolaryngology-Head and Neck Surgery, Changhai Hospital, Second Military Medical University, Shanghai, China. Tel: 011861376430-3602; E-mail: zheng_hl2004@163.com

References

[1] Mao L, Hong WK and Papadimitrakopoulou VA. Focus on head and neck cancer. Cancer Cell 2004; 5: 311-316.

[2] Weber GF. The metastasis gene osteopontin: a candidate target for cancer therapy. Biochim Biophys Acta 2001; 1552: 61-85.

[3] Rangaswami H, Bulbule A and Kundu GC. Osteopontin: role in cell signaling and cancer progression. Trends Cell Biol 2006; 16: 79-87.

[4] Rittling SR and Chambers AF. Role of osteopontin in tumour progression. Br J Cancer 2004; 90: 1877-1881.

[5] Wai PY and Kuo PC. Osteopontin: regulation in tumor metastasis. Cancer Metastasis Rev 2008; 27: 103-118.

[6] Katagiri YU, Sleeman J, Fujii H, Herrlich P, Hotta H, Tanaka K, Chikuma S, Yagita H, Okumura K, Murakami M, Saiki I, Chambers AF and Uede T. CD44 variants but not CD44s cooperate with beta1-containing integrins to permit cells to bind to osteopontin independently of arginine-glycine-aspartic acid, thereby stimulating cell motility and chemotaxis. Cancer Res 1999; 59: 219-226.

[7] Weber GF, Ashkar S, Glimcher MJ and Cantor H. Receptor-ligand interaction between CD44 and osteopontin (Eta-1). Science 1996; 271: 509-512.

[8] Crawford HC, Matrisian LM and Liaw L. Distinct roles of osteopontin in host defense activity and tumor survival during squamous cell carcinoma progression in vivo. Cancer Res 1998; 58: 5206-5215.

[9] Coppola D, Szabo M, Boulware D, Muraca P, Alsarraj M, Chambers AF and Yeatman TJ. Correlation of osteopontin protein expression and pathological stage across a wide variety of

tumor histologies. Clin Cancer Res 2004; 10: 184-190.

[10] Gillespie MT, Thomas RJ, Pu ZY, Zhou H, Martin TJ and Findlay DM. Calcitonin receptors, bone sialoprotein and osteopontin are expressed in primary breast cancers. Int J Cancer 1997; 73: 812-815.

[11] Kim JH, Skates SJ, Uede T, Wong KK, Schorge JO, Feltmate CM, Berkowitz RS, Cramer DW and Mok SC. Osteopontin as a potential diagnostic biomarker for ovarian cancer. JAMA 2002; 287: 1671-1679.

[12] Fedarko NS, Jain A, Karadag A, Van Eman MR and Fisher LW. Elevated serum bone sialoprotein and osteopontin in colon, breast, prostate, and lung cancer. Clin Cancer Res 2001; 7: 4060-4066.

[13] Hotte SJ, Winquist EW, Stitt L, Wilson SM and Chambers AF. Plasma osteopontin: associations with survival and metastasis to bone in men with hormone-refractory prostate carcinoma. Cancer 2002; 95: 506-512.

[14] Shimada Y, Watanabe G, Kawamura J, Soma T, Okabe M, Ito T, Inoue H, Kondo M, Mori Y, Tanaka E and Imamura M. Clinical significance of osteopontin in esophageal squamous cell carcinoma: comparison with common tumor markers. Oncology 2005; 68: 285-292.

[15] Li Y, Li L, Wang JT, Kan X and Lu JG. Elevated content of osteopontin in plasma and tumor tissues of patients with laryngeal and hypopharyngeal carcinoma associated with metastasis and prognosis. Med Oncol 2012; 29: 1429-1434.

[16] El-Tanani MK, Campbell FC, Kurisetty V, Jin D, McCann M and Rudland PS. The regulation and role of osteopontin in malignant transformation and cancer. Cytokine Growth Factor Rev 2006; 17: 463-474.

[17] Meerovitch K, Bergeron F, Leblond L, Grouix B, Poirier C, Bubenik M, Chan L, Gourdeau H, Bowlin T and Attardo G. A novel RGD antagonist that targets both alphavbeta3 and alpha-5beta1 induces apoptosis of angiogenic endothelial cells on type I collagen. Vascul Pharmacol 2003; 40: 77-89.

[18] Thompson L. World Health Organization classification of tumours: pathology and genetics of head and neck tumours. Ear Nose Throat J 2006; 85: 74.

[19] Bache M, Reddemann R, Said HM, Holzhausen HJ, Taubert H, Becker A, Kuhnt T, Hansgen G, Dunst J and Vordermark D. Immunohistochemical detection of osteopontin in advanced head-and-neck cancer: prognostic role and correlation with oxygen electrode measurements, hypoxia-inducible-factor-1alpha-related markers, and hemoglobin levels. Int J Radiat Oncol Biol Phys 2006; 66: 1481-1487.

[20] Weidner N. Current pathologic methods for measuring intratumoral microvessel density within breast carcinoma and other solid tumors. Breast Cancer Res Treat 1995; 36: 169-180.

[21] Jemal A, Siegel R, Ward E, Murray T, Xu J, Smigal C and Thun MJ. Cancer statistics, 2006. CA Cancer J Clin 2006; 56: 106-130.

[22] Licitra L, Bernier J, Grandi C, Locati L, Merlano M, Gatta G and Lefebvre JL. Cancer of the larynx. Crit Rev Oncol Hematol 2003; 47: 65-80.

[23] Eto M, Kodama S, Nomi N, Uemura N and Suzuki M. Clinical significance of elevated osteopontin levels in head and neck cancer patients. Auris Nasus Larynx 2007; 34: 343-346.

[24] Staibano S, Merolla F, Testa D, Iovine R, Mascolo M, Guarino V, Castellone MD, Di Benedetto M, Galli V, Motta S, Melillo RM, De Rosa G, Santoro M and Celetti A. OPN/CD44v6 overexpression in laryngeal dysplasia and correlation with clinical outcome. Br J Cancer 2007; 97: 1545-1551.

[25] Elbashir SM, Lendeckel W and Tuschl T. RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev 2001; 15: 188-200.

[26] Meister G and Tuschl T. Mechanisms of gene silencing by double-stranded RNA. Nature 2004; 431: 343-349.

[27] Van den Haute C, Eggermont K, Nuttin B, Debyser Z and Baekelandt V. Lentiviral vector-mediated delivery of short hairpin RNA results in persistent knockdown of gene expression in mouse brain. Hum Gene Ther 2003; 14: 1799-1807.

[28] Lebedev TD, Spirin PV and Prassolov VS. Transfer and Expression of Small Interfering RNAs in Mammalian Cells Using Lentiviral Vectors. Acta Naturae 2013; 5: 7-18.

[29] hahneh FZ, Baradaran B, Zamani F and Aghebati-Maleki L. Tumor angiogenesis and anti-angiogenic therapies. Hum Antibodies 2013; 22: 15-19.

[30] Bellou S, Pentheroudakis G, Murphy C and Fotsis T. Anti-angiogenesis in cancer therapy: Hercules and hydra. Cancer Lett 2013; 338: 219-228.

[31] Hsu HW, Wall NR, Hsueh CT, Kim S, Ferris RL, Chen CS and Mirshahidi S. Combination anti-angiogenic therapy and radiation in head and neck cancers. Oral Oncol 2014; 50: 19-26.

[32] Lionello M, Staffieri A and Marioni G. Potential prognostic and therapeutic role for angiogenesis markers in laryngeal carcinoma. Acta Otolaryngol 2012; 132: 574-582.

[33] Simonetti O, Lucarini G, Rubini C, Goteri G, Zizzi A, Staibano S, Campanati A, Ganzetti G, Di Primio R and Offidani A. Microvessel density and VEGF, HIF-1alpha expression in primary oral melanoma: correlation with prognosis. Oral Dis 2013; 19: 620-627.