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Original Article

Expressions of vascular endothelial growth factor (VEGF), platelet-derived growth factor (PD-ECGF) and cyclooxygenase-2 (COX-2) in laryngeal squamous cell carcinoma

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Abstract: The aim of this study was to investigate the expressions, significance and correlations of vascular endothelial growth factor (VEGF), platelet-derived growth factor (PD-ECGF) and cyclooxygenase-2 (COX-2) in laryngeal squamous cell carcinoma (LSCC). The expressions of VEGF, PD-ECGF and COX-2 were determined with S-P immunohistochemical analysis in 76 primary LSCC patients and 15 patients with polyp of larynx. The associations of their expressions with pathological and clinical features were statistically analyzed. The positive expression rates of VEGF (63.16%), PD-ECGF (57.89%) and COX-2 (51.32%) in LSCC were significantly higher than those in polyp of larynx ($P<0.01$). The positive expressions of VEGF, PD-ECGF and COX-2 in LSCC were correlated with the tumor differentiation and invasive depth ($P<0.05$). The positive expression of VEGF in LSCC was related to lymph node metastasis ($P<0.01$). There were positive correlations between the expressions of VEGF and COX-2 ($P<0.05$), PD-ECGF and COX-2 ($P<0.01$). VEGF, PD-ECGF and COX-2 might play key roles in LSCC. All might have close relations in angiogenesis and might provide new targets for LSCC therapy.

Keywords: Vascular endothelial growth factor, platelet-derived growth factor, cyclooxygenase-2, laryngeal neoplasms, carcinoma, squamous cell, immunohistochemistry

Introduction

Laryngeal squamous cell carcinoma (LSCC) was one of the common head and neck cancers in recent years, its incidence rapidly grew [1-3]. Although the advances in surgical techniques, radiochemotherapy and immunotherapy made the overall survival rate of LSCC patients greatly improved, postoperative recurrence and metastasis were still the main problems that affected the overall survival rate [4]. Numerous studies in the last 10 years showed that the abnormal expressions of vascular endothelial growth factor (VEGF), platelet-derived growth factor (PD-ECGF) and cyclooxygenase-2 (COX-2) were found in the studies such human malignancies as ovarian cancer, breast cancer, esophageal cancer, gastric cancer, bladder cancer and cell lines [5-11]. But their relationships with the biological behaviors of LSCC were still not clear yet. This study used SP immunohistochemical assay to detect the expressions of VEGF, PD-ECGF and COX-2 in LSCC, aiming to explore their relationships with the differentiation, invasion and lymph node metastasis of LSCC.

Methods

Materials

76 surgically-resected primary LSCC specimens were collected from The Second Clinical Medical College of Fujian Medical University from 2008 to 2013. The inclusion criteria was set as that the LSCC patients should not receive any anti-tumor therapy before surgery, and had complete clinical data and pathological data.

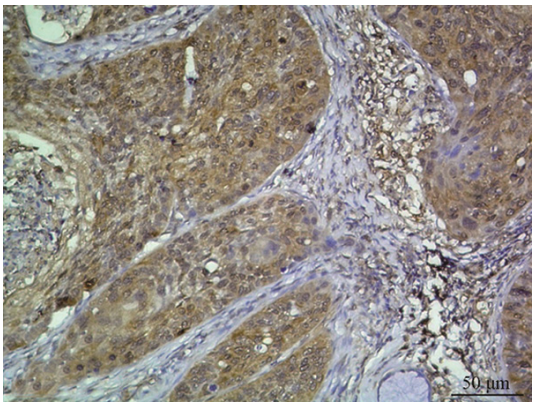

Figure 1. The positive VEGF expression in LSCC was located inside cytoplasm (SP method ×200). (negative staining results in LSCC or polyps should also be included).

Grouping

Among the 76 LSCC cases, 73 cases were males and 3 cases were females, aged 41 to 83 years old (the average age was 60.3 years old, with the median age as 62.7 years old). All cases were confirmed by pathological diagnosis, and grouped according to the LSCC TNM classification of UICC [1]: ① according to the tumor invasion degrees: $T_{1,2}$ group: 30 cases, $T_{3,4}$ group: 46 cases; ② according to the histological grades: highly-differentiated group: 34 cases, poorly-differentiated group: 42 cases; ③ existed metastasis or not: lymph node metastasis group ($N_{1\sim3}$): 15 cases, without lymph node metastasis group (N_0): 61 cases. The control group: 15 patients with pathologically confirmed laryngeal polyp were selected as the control group, including 8 males and 7 females, aged 25 to 48 years old (the average age was 36.5 years old, with the median age as 38.1 years old). This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of Fujian Medical University. Written informed consent was obtained from all participants.

Experimental methods

The above specimens were detected the expressions of VEGF, PD-ECGF and COX-2 protein by immunohistochemical SP method, the specific steps were in accordance with SP kit instructions. Reagent sources: mouse anti-human VEGF monoclonal antibody (sc-7269), rabbit anti-human PD-ECGF polyclonal antibody (sc-128) and goat anti-human COX-2 polyclonal antibody (sc-1746) were purchased from Santa Cruz (USA), and the SP immunohistochemical hypersensitivity staining kit and DAB staining kit were purchased from Beijing Zhongshan Biotechnology Co., Ltd.

Determination of results

Two pathologists observed the immunohistochemical staining results under microscope independently. The positive staining of VEGF, PD-ECGF and COX-2 was defined as the brown granular staining inside cytoplasm. Each slice was randomly selected 10 representative 400× high-power fields for the observation and counting, each field counted 100 tumor cells. The semi-quantitative integration was performed according to the literature method [2], and the classification was based on the staining intensities and the percentages of positive cells: staining intensity (non-stained, weak, medium, strong) and percentage of positive cells (0%, 1 to 25%, 26 to 50%, >50%) were respectively integrated as 0, 1, 2 and 3 points. When the sum of two scores was 0 to 2 points, the result was recorded as the negative expression of VEGF and COX-2; while ≥3 points was then recorded as positive expressions of VEGF and COX-2.

Statistical methods

The data were analyzed with SPSS version 17.0 statistical package. The correlations of VEGF, PD-ECGF and COX-2 expressions with LSCC clinicopathological parameters used the Chi-square test (χ^2 test), the correlation analysis among the indicators used the Kappa method for the consistency test, with $P<0.05$ considered as statistical significance.

Results

VEGF expression in LSCC

The positive expression of VEGF in LSCC were mainly located inside cytoplasm, showing obvious brown granules, the positive rate was 63.16% (48/76), there was no expression inside nuclei (**Figure 1**); in the control group, the positive signals of VEGF were mainly exhibited in cytoplasm, but the staining was weak,

Table 1. Expression of VEGF, PD-ECGF, and COX-2 in laryngeal carcinoma and laryngeal polyp

	Laryngeal carcinoma (n=76)			Laryngeal polyp (n=15)			χ^2 value	*P* value
	+	-	Positive ratio (%)	+	-	Positive ratio (%)		
VEGF	48	28	63.16	3	12	20	9.472	<0.01
PD-ECGF	44	32	57.89	2	13	13.33	9.952	<0.01
COX-2	39	37	51.32	0	15	0	11.456	<0.01

Table 2. Relationship between expression of VEGF and clinical and pathological features in patients with laryngeal carcinoma

	n	VEGF expression			χ^2 value	*P* value
		+	-	Positive ratio (%)		
Depth of invasion					11.424	<0.01
T_1~T_2	30	12	18	40.00		
T_3~T_4	46	36	10	78.26		
Differentiated degree					12.775	<0.01
Well	34	14	20	41.18		
Poor-moderate	42	34	8	80.95		
Lymph node metastasis					27.313	<0.01
N_0	61	34	27	55.74		
$N_{1\sim3}$	15	14	1	93.33		

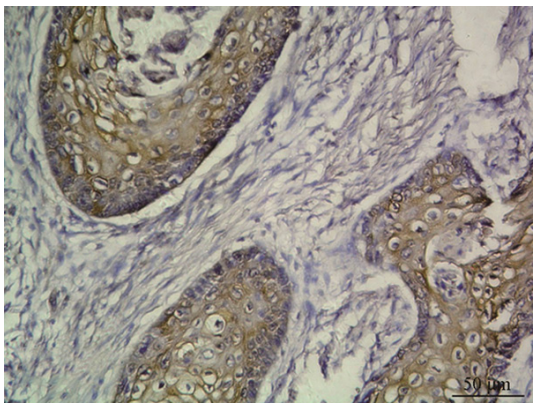

Figure 2. The positive PD-ECGF expression in LSCC was located inside cytoplasm (SP method ×200). (negative staining results in LSCC or polyps should also be included).

meanwhile, various degrees of nuclear expression could be seen, the total positive rate was 20% (3/15). The positive VEGF expression in LSCC was higher than the control group, and the difference was statistically significant (*P*<0.01) (**Table 1**).

Correlations of VEGF expression and clinicopathological parameters of LSCC

The VEGF expression was closely related to the invasion depth and differentiation degrees, the deeper the invasion, the worse the differentiation degree, the stronger the expression (*P*<0.01); the expression of VEGF in the patients with lymph node metastasis was significantly higher than those without lymph node metastasis (*P*<0.01) (**Table 2**).

PD-ECGF expression in LSCC

The PD-ECGF expression in LSCC was mainly located inside cytoplasm, showing obvious brown granules, the positive rate was 57.89% (44/76), while there was no expression inside nuclei (**Figure 2**); in the control group, the positive signals of VEGF were also mainly exhibited in cytoplasm, but the staining was weak, the total positive rate was 13.33% (2/15), and no expression was inside nuclei. The positive PD-ECGF expression in LSCC was higher than the control group, and the difference was statistically significant (*P*<0.01) (**Table 1**).

Correlations of PD-ECGF expression and clinicopathological parameters of LSCC

The PD-ECGF expression was closely related to the invasion depth and differentiation degrees, the deeper the invasion, the worse the differentiation degree, the stronger the expression (*P*<0.05); while the no significant correlation existed between the expression of PD-ECGF and the lymph node metastasis (*P*>0.05) (**Table 3**).

COX-2 expression in LSCC

The COX-2 expression in LSCC was mainly located inside cytoplasm, showing obvious brown granules, the positive rate was 51.32% (39/76), while there was no expression inside nuclei (**Figure 3**). There was no positive expression of COX-2 in the control group. The positive COX-2 expression in LSCC was higher than the

Table 3. Relationship between expression of PD-ECGF and clinical and pathological features in patients with laryngeal carcinoma

	n	PD-ECGF expression			χ^2 value	*P* value
		+	-	Positive ratio (%)		
Depth of invasion					9.163	<0.01
T_1~T_2	30	11	19	36.67		
T_3~T_4	46	33	13	71.74		
Differentiated degree					4.790	<0.05
Well	34	15	19	44.12		
Poor-moderate	42	29	13	69.05		
Lymph node metastasis					0.159	>0.05
N_0	61	36	25	59.02		
$N_{1\sim3}$	15	7	8	53.33		

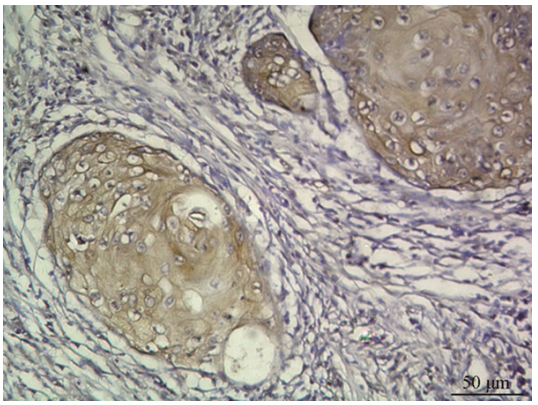

Figure 3. The positive COX-2 expression in LSCC was located inside cytoplasm (SP method ×200). (negative staining results in LSCC or polyps should also be included).

control group, and the difference was statistically significant (*P*<0.01) (**Table 1**).

Correlations of COX-2 expression and clinicopathological parameters of LSCC

The COX-2 expression was closely related to the invasion depth and differentiation degrees, the deeper the invasion, the worse the differentiation degree, the stronger the expression (*P*<0.05); while the no significant correlation existed between the expression of COX-2 and the lymph node metastasis (*P*>0.05) (**Table 4**).

Correlations among expressions of VEGF, PD-ECGF and COX-2 in LSCC

The correlation analysis between two proteins showed that COX-2 and VEGF, as well as COX-2 and PD-ECGF, had the expression correlations in LSCC, and the significance was statistical (*P*<0.05); while the expressions of VEGF and PD-ECGF had no significant correlation (*P*>0.05) (**Tables 5-7**).

Discussion

Expression and significance of VEGF in LSCC

VEGF was also known as vascular permeability factor (VPF). It acted on vascular endothelial cells via binding to its specific receptors, thus resulting in the deformation, migration and disassociation of endothelial cells and enhancing the blood vessels' permeability. Therefore, it would not only directly affect the neovascular formation, but also closely related to the invasion and metastasis of tumor cells [12]. Studies had shown that the cancer patients with strong expression of VEGF were accompanied with more blood vascular, lymph node and hepatic metastases, thus their disease conditions would progress faster than the patients with weak expression of VEGF, their recurrence would be rapid, and the postoperative survival time would be shorter. In recent years, numerous studies about the relationships of VEGF and various tumors such as human breast cancer, colon cancer, renal cell carcinoma, bladder cancer and malignant glioma showed that [5, 6], the VEGF expression levels inside tumors were significantly higher than those in normal tissues, and positively correlated with such poor clinical prognosis as tumorous vascularization degrees, lymph node metastasis and recurrence, it was considered that VEGF could be used as the poor prognostic indicator of many solid malignancies [13]. The experimental data of this study showed that the positive expression rate of VEGF in LSCC was 63.16%, significantly higher than that in the control group, and the difference was statistically significant (*P*<0.01); and the biological study showed that the VEGF expression was closely related to the invasion depth, differentiation degrees and lymph node metastasis of LSCC, the patients with deeper invasion, poorer tissue differentiation and lymph node metastasis exhibited high VEGF expression in their cancer tissues.

Table 4. Relationship between expression of COX-2 and clinical and pathological features in patients with laryngeal carcinoma

	n	COX-2 expression			χ^2 value	*P* value
		+	-	Positive ratio (%)		
Depth of invasion					9.0145	<0.01
T_1~T_2	30	9	21	30.00		
T_3~T_4	46	30	16	65.22		
Differentiated degree					6.321	<0.05
Well	34	12	22	35.29		
Poor-moderate	42	27	15	64.29		
Lymph node metastasis					0.162	>0.05
N_0	61	32	29	52.46		
$N_{1\sim3}$	15	7	8	46.67		

Table 5. Relationship between expression of VEGF and PD-ECGF in laryngeal carcinoma

VEGF expression	PD-ECGF expression		Kappa value	*P* value
	-	+		
-	15	13	0.176	>0.05
+	17	31		

Table 6. Relationship between expression of VEGF and COX-2 in laryngeal carcinoma

VEGF	COX-2 expression		Kappa value	*P* value
	-	+		
-	19	9	0.285	<0.05
+	18	30		

Table 7. Relationship between expression of PD-ECGF and COX-2 in laryngeal carcinoma

PD-ECGF expression	COX-2 expression		Kappa value	*P* value
	-	+		
-	27	5	0.604	<0.01
+	10	34		

Expression and significance of PD-ECGF in LSCC

PD-ECGF was a potent mitogen, as well as an important vascular growth factor, secreted by platelets, tissue cells and some tumor cells. PD-ECGF exhibited important effects of growth promotion and stability maintenance in normal tissues, its overexpression was closely associated with growth, proliferation and angiogenesis of tumors [7]. The results showed that besides inducing the migration of vascular endothelial cells and smooth muscle cells, the tumor cells-released PD-ECGF could also stimulate the growth of these cells, thus playing a direct role towards the tumor angiogenesis, it had been demonstrated that the PD-ECGF expression in a variety of tumor tissues was significantly higher than the normal tissues [8]. The results of this study showed that the positive expression rates of PD-ECGF in LSCC and laryngeal polyps were 57.89% (44/76) and 13.33% (2/15), respectively, exhibiting statistically significant difference (P<0.01), indicating that PD-ECGF might be involved in the occurrence of LSCC. The relationship analysis towards the biological characteristics of LSCC showed that the VEGF expression was positively correlated with the invasion depth and differentiation degrees of tissues, the deeper the tumor's invasion, the worse the differentiation, the stronger the PD-ECGF expression (P<0.05). We found that the PD-ECGF expression in LSCC associated with lymph node metastasis was higher than those without lymph node metastasis, but the difference was not statistically significant (P>0.05), further large-sample studies should be performed in order to further determine their relationships.

Expression and significance of COX-2 in LSCC

COX-2 was also known as prostaglandin endoperoxide synthase, it was the key rate-limiting enzyme in transforming the arachidonic acid into prostaglandins, as an important inflammatory mediator, prostaglandin had various functions as promoting cell proliferation, inhibiting apoptosis and stimulating angiogenesis, etc. Currently, it had been found that COX had at least three kinds of isoenzymes, among which COX-2 was the inducer-type gene, and could not be expressed under resting state, when the cells were stimulated by growth factors, cytokines, hormones, intracellular toxins and others, its expression could be rapidly enhanced, thus promoting the tumor angiogenesis, maintaining the growth of tumor tissues and promoting the invasion and metastasis of tumors [14]. A large number of studies had found that COX-2

was overexpressed in a variety of solid tumors, such as breast cancer, prostate cancer and liver cancer [9-11]. The increased expression levels of COX-2 could promote tumor growth and induce tumor resistance, so it could be used as an indicator of poor prognosis [15]. The evidence suggested that COX-2 had high correlations with tumors, and might be involved in tumorigenesis and development through a variety of mechanisms. Among the 76 LSCC cases detected in this study, the positive expression rate of COX-2 protein was 51.31%, significantly higher than the benign laryngeal polyps (the latter had no expression), suggesting that the expression of COX-2 protein might be associated with the occurrence of LSCC. Among the 39 cases that had the positive expressions of COX-2 protein, the positive cells mainly exhibited nidal appearance, and mainly distributed in the tumor cell nest at the edge of primary tumors, suggesting that the expression level of COX-2 was related with the proliferation activities of cancer cells. The immunohistochemistry showed that the positive rate in the poorly differentiated group was higher than the highly-differentiated group ($P<0.05$), and the positive rate in the deep-invasion group was significantly higher than the superficial-invasion group ($P<0.01$). But the difference between the lymph node metastasis or not was not significant ($P>0.05$), the author analyzed that it might be related with the fact that there was great difference in the patient numbers with or without lymph node metastasis in this study, the future studies should expand the sample size to further observe the relationships between COX-2 and lymph node metastasis of LSCC.

Expression correlations of VEGF, PD-ECGF and COX-2 in LSCC

The tumor growth and metastasis was a complex process decided by many factors such as angiogenesis, host's immune functions, and tumor cell invasion, etc. Angiogenesis was an essential process in growth and metastasis of primary tumors, and the key step in tumor growth and canceration [16]. The intratumoral neonatal microvessels could not only provide essential nutrients towards the tumor growth, transport their metabolites, but also provide a channel for tumor cells to enter the circulation system. The neonatal microvessels could also promote the proliferation of tumor cells by endothelial cells-secreted growth factors. Studies had shown that [17, 18], COX-2 played an important role in tumor angiogenesis, the non-specific and specific COX-2 inhibitors could significantly inhibit the angiogenesis. Certain foreign data had confirmed that COX-2 could upregulate the VEGF expression, promote the tumor angiogenesis, inhibiting the expression of COX-2 could also inhibit the VEGF expression [19, 20]. Timoshenko reported in his studies of breast cancer that COX-2 could promote the synthesis of VEGF inside cancer cells, when silenced the COX-2 gene expression using the RNA interference method, it could cause the significant reduction of VEGF mRNA [21]. Su showed that in lung cancer cell lines, COX-2 might upregulate the expression of VEGF through its metabolite prostaglandin E2 (PGE2) [22].

Through analyzing the correlations of VEGF, PD-ECGF and COX-2, we found that in LSCC, the expressions of COX-2 and VEGF were significantly positively correlated ($P<0.05$), supporting the above view, suggesting that the involvement of COX-2 in tumor angiogenesis was achieved by affecting the expression of tumor vascular endothelial growth factor. COX-2 could upregulate the VEGF expression through it-synthesized PGE2, inhibiting COX-2 could inhibit the expression of VEGF.

The study found that VEGF, PD-ECGF, transforming growth factor-β (TGF-β) and basic fibroblast growth factor (bFGF) might be involved in the growth and metastasis of some tumors. And it had been identified that VEGF and PD-ECGF were the major cell generation inducing factors in human ovarian cancer, malignant glioblastoma and breast cancer, besides related to the angiogenesis of these tumors, PD-ECGF was also involved in the tumor metastasis. PD-ECGF could also induce the transcription and secretion of vascular endothelial growth factor, thus indirectly promoting the tumor angiogenesis [8]. The correlation analysis of this experiment also showed that the protein expressions of COX-2 and PD-ECGF were positively correlated ($P<0.05$), and the regional consistency of expressions of these 2 proteins was obvious, mainly distributed in the cancer cell nests at the edge of primary tumors, suggesting that the expression levels of these 2 proteins were related to the proliferation activities of cancer cells.

In summary, the expression and correlation study of VEGF, PD-ECGF and COX-2 in LSCC showed that these three factors were closely related to the angiogenesis and biological behaviors of LSCC, and might possibly be used to determine the biological behaviors, metastatic potential and prognosis of LSCC. Eliminating the stimulation of tumor cells to the angiogenesis could be realized by applying the angiogenesis inhibitors to block the tumor angiogenesis, thus reducing the proliferation, recurrence and metastasis of tumors, and it had become a new anti-tumor direction. Assessing the tumor angiogenesis would be beneficial towards determining the malignant trend of precancerous lesions and the prognosis of tumors, screening patients with high-risk of recurrence or metastasis, and guiding clinics for the early prevention and treatment.

Disclosure of conflict of interest

None.

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