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淋巴管新生在頭頸部癌症轉移中所扮演的角色

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題目：淋巴管新生在頭頸部癌症轉移中所扮演的角色

The role of lymphangiogenesis in head and neck cancer metastasis

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摘要

雖然血管新生在癌症轉移中扮演著重要的角色，也受到廣泛地研究，但近年來淋巴管新生被認為可能更具重要性，臨床病理的資料也顯示淋巴管可能是實體腫瘤轉移的第二步，然而目前有關於淋巴管新生的研究仍不完備。腫瘤細胞侵入淋巴管進而產生淋巴結轉移，會顯著地影響癌症病患的預後。頭頸部腫瘤常伴有淋巴結轉移的現象，因此有關淋巴管新生和頭頸部腫瘤轉移的研究就更具意義。一些關於淋巴管新生的實驗顯示，在與血管新生有關的血管內皮細胞生長因子（vascular endothelial growth factor, VEGF）家族中，新發現的C型血管上皮細胞生長因子（VEGF-C）與淋巴管新生有關。然而在血管新生中對VDGF具有調控作用的第二型環氧酵素（cyclooxygenase-2, COX-2）及第六介白質（Interleukin-6, IL-6）是否也可能調控淋巴管新生仍有待研究。為了解這些因子在頭頸部鱗狀上皮癌（HNSCC）中的表現與患者預後的關係，我們以RT-PCR的方式檢測HNSCC腫瘤中VEGF-C、COX-2及IL-6之mRNA的表現，以免疫組織學染色觀察這些因子在腫瘤組織中分佈的情形，再將上述表現與臨床疾病表現的相關性作分析。實驗已經證實所使用的RT-PCR及免疫組織學染色可以偵測出腫瘤組織中VEGF-C、COX-2及IL-6的表現，結果顯示，嚼食檳榔者會在腫瘤組織中表現較高的淋巴管新生因子，這可能與其癌症轉移相關，尋找針對淋巴管新生作用的藥物，可能可以作為治療癌症轉移的相關輔助療法。

關鍵字： 淋巴管新生、頭頸部鱗狀上皮癌（HNSCC）、C型血管內皮細胞生長因子（VEGF-C）、第二型環氧酵素（COX-2）、第六介白質（IL-6）

While angiogenesis is crucial for the progression of tumors, the more recent concept is that lymphangiogenesis is suspected as being of greater importance in relation to metastatic spread. Indeed, clinicopathological data suggests that the lymphatics are an initial route for the spread of solid tumors. Nevertheless, in contrast to blood vessel angiogenesis, the mechanism of new lymphatic vessel formation in human cancer, i.e. lymphangiogenesis, is still relatively unclear. Since the invasion of lymphatic vessels by tumor cells and subsequent development of lymph node metastases would significantly influence the prognosis of cancer patients, it is meaningful to study the relationship between the lymphangiogenesis and the head and neck cancer, as the latter is frequently associated with cervical lymph node metastasis. Studies on lymphangiogenesis revealed that vascular endothelial growth factor (VEGF)-C, a novel member of the VEGF family of angiogenic growth factors, could stimulate the growth of lymphatic vascular endothelium in vivo. However, whether important modulators in angiogenesis, cyclooxygenase-2 (COX-2) and interleukin-6 (IL-6), may also play a role in the signaling pathway that leads to lymphangiogenesis still needs to be clarified. To better understand the expression of these factors and their relationship with the prognosis of head and neck squamous cell carcinoma (HNSCC)
patients, this study examined the expressions of VEGF-C, COX2, and IL-6 mRNA and protein levels in 100 surgically resected HNSCC tumors. All 3 kinds of mRNA were detected by the RT-PCR method, and protein levels were studied by immunohistochemical staining and graded by a staining score. We correlated these markers with tumor size, histology, and clinical outcome. Results have shown that both RT-PCR and immunohistochemical staining could detect the expressions of VEGF-C, COX2, and IL-6 in HNSCC tumors. Patients who had chew betel nuts for long time had higher expression of these lymphangiogenesis factors, which might be related to the metastasis. For those patients who are likely to have metastasis diseases, we can use more aggressive therapy to treat the diseases. In addition, the development of therapeutic molecules that are able to target lymphangiogenesis, possibly in combination with traditional therapeutic methods, may signal a quantum leap in cancer therapy or prevention of metastasis.

Key words: lymphangiogenesis, head and neck squamous cell carcinoma (HNSCC), vascular endothelial growth factor-C (VEGF-C), cyclooxygenase-2 (COX-2), interleukin-6 (IL-6)

Introduction

Head and neck cancer is a major, worldwide cause of morbidity and mortality. As long as the neoplasm is confined to its organ of origin, the patient can be cured through surgical removal of the tumor mass. Unfortunately, many cancers metastasize to other sites in the body, and metastasis is the leading cause of death in cancer patients (1-3). In principle, cancer cells can spread within the body by different mechanisms, such as direct invasion of surrounding tissues (per continuitatem), spread via the blood vascular system (hematogenous metastasis) and spread via the lymphatic system (lymphatic metastasis) (4). Tumor cells can invade either the blood or lymphatic vessels to access the general circulation and then establish themselves in other tissues. Clinicopathological data suggest that the lymphatics are an initial route for the spread of solid tumors (5). Infiltration of lymphatic vessels by tumor cells has been found at the periphery of many experimental and human tumors, and the lymphatic system has been recognized as a conduit for tumor cell dissemination (6). Though the significance of angiogenesis for tumor progression has been well documented, the molecular mechanisms regulating the growth and function of lymphatic vessels are largely unknown.

Vascular endothelial growth factors, first identified in 1989 (7), are well-known angiogenic agents (8) and targets for anti-cancer therapies (9). Now it appears that VEGF-C (10), one recently-cloned member of the vascular endothelial growth factor (VEGF) family, is also involved in developmental and tumor-induced lymphangiogenesis. VEGF signals through two tyrosine kinase receptors, VEGFR-1 and VEGFR-2, which are expressed predominantly but not exclusively on vascular endothelial cells (11). As neither VEGFR-1 nor VEGFR-2 appear to be highly expressed in lymphatic endothelium, it was not surprising that a third VEGF receptor, VEGFR-3, was found to be predominantly expressed on lymphatic vessels during development (12). What was surprising, however, was that VEGF was not found to bind to VEGFR-3. Instead, VEGF-C was discovered to be ligand for VEGFR-3 (10). Research groups provide direct evidence that VEGF-C is not only important regulator of lymph vessel growth (lymphangiogenesis) in vivo (13,14) but also enhance lymphatic metastasis (15-17). Using experimental approaches, Mäkinen et al. (15), Skobe et al.(16), as well as Mandriota et al. (17) demonstrate an important role of VEGFR-3 and its ligands, VEGF-C, in developmental and tumor-induced lymphangiogenesis. In normal adult human tissues, the VEGF-C receptor VEGFR-3 (FLT-4) is predominantly expressed by lymphatic endothelia (12,18). Expression of VEGF-C occurs in a variety of human tumors such as breast (19,20), colon (21,22), lung (23,24), thyroid (25-27), gastric
Moreover, expression of VEGF-C mRNA has recently been shown to correlate with the rate of metastasis to lymph nodes in breast (19), colorectal (22), gastric (28), thyroid (26,27), lung (24) and prostate (31) cancers. To date, however, lymphangiogenesis has not been causally linked to tumor metastasis. Cyclooxygenase-2 (COX-2) enzyme catalyzes the synthesis of prostaglandins. COX-2 is an immediate-early response gene induced by inflammation, growth factors, tumor promoters, oncogenes, and carcinogens (32,33). Increased levels of COX-2 may contribute to carcinogenesis by modulating xenobiotic metabolism, apoptosis, immune surveillance, and angiogenesis. Any significant increase in tumor mass must be preceded by an increase in vascular supply to deliver nutrients and oxygen to the tumor (34). Recently, levels of COX-2 were found to correlate with both VEGF expression and tumor vascularization in HNSCC (35). This finding in human tissues is consistent with prior evidence that overexpression of COX-2 in epithelial cells led to enhanced production of VEGF and the formation of capillary-like networks (36). Although COX-2 contributes to the regulation of angiogenesis, its role in lymphangiogenesis is not clear.

IL-6 is a secreted, multifunctional glycoprotein. Through binding to α-chain (IL-6-R, gp80) and subsequently recruiting the β-chain (gp130) of the receptor, IL-6 performs various biological functions (37). The diversity of IL-6 signaling mediated via gp130 explains its functional pleiotrophy (38). IL-6 regulates inflammatory reactions, immune responses, hepatic acute-phase protein synthesis, and several other important physiological processes (39). Interestingly, the influence of IL-6 in human cancers is varied depending on the cell types. For example, IL-6 has been demonstrated to promote growth of multiple myeloma, Kaposi's sarcoma, and prostatic cancer cells, while inhibiting the proliferation of lung and breast cancer cells (40-44). Previous investigations have confirmed that IL-6 is important in both physiological and pathological angiogenesis (45-47). Additionally, recent study supports the hypothesis that IL-6 facilitates tumorigenesis of cervical cancer via VEGF-mediated angiogenesis (48). Nevertheless, whether IL-6 could regulate the expression of VEGF-C and what is its role in lymphangiogenesis still need to be clarified.

Inhibition of angiogenesis is currently considered one of the most promising therapeutic strategies to inhibit cancer growth because it presumably can act on any tumor type, does not induce resistance of tumor cells (and can therefore be used in repeated therapeutic cycles) and has little effect on normal tissues. It now needs to be determined whether the same holds true for tumor lymphangiogenesis.

Metastases of head and neck cancers occur frequently through the lymphatic system, and the extent of lymph node involvement is a key prognostic factor for the diseases. In this study, we will conduct a systematic analysis of VEGF-C, COX-2 and IL-6 expressions and will try to find the correlation between their expressions, lymphatic metastases and patient survival. The findings of this study will help us understand whether lymphangiogenesis could be a focal point of anti-cancer research.

Materials and Methods

Patient population

One hundred patients with surgically resected SCC head and neck tumors were included in this study, all of which were collected retrospectively from the National Taiwan University Hospital. The epidemiological data including demographics, family history, occupational exposure, medical history, and histopathological data on these patients were analyzed.

Antibodies and reagents

Recombinant human IL-6 and human polyclonal VEGF-C antibody were purchased from R&D Systems (Minneapolis, MN). COX-2 antibody, β-actin, and secondary horseradish peroxidase-conjugated...
antibody were obtained from Santa Cruz Biotechnology.

**RT-PCR**
The RT-PCR was performed using an RT-PCR kit (LIFE Technologies). Two micrograms of total RNA were used for RT reaction. The sequences of the primers are as follows; human (h)VEGF-C (58°C): sense 5'-CCT GGT GGA CAT TTT CCA GGA GTA CC-3' and antisense 5'-CTC ACC GCC TCG GCT TGT CA-3'; hIL-6 (56°C): sense 5'-ATG T AG CCG CCC CAC ACA GA-3' and antisense 5'-CAA TCA TCT TTT TCA GCC AT-3'; COX-2: 5'-TTC AAA TGA GAT TGT GGG AAA AT and antisense 3'-TTC TAT GAG TCC GTC TCT ACT AGA; GADPH (55°C): sense 5'-GAA GGT GAA GGT CGG AGT CAA-3' and antisense 5'-GCA GAG GGG GCA GAG ATG AT-3'. Primers were used at a final concentration of 0.5µM. Reaction mixture was first denatured at 94 °C for 5 min. The PCR condition was 94 °C for 30 sec, 55 °C for 45 sec, and 72 °C for 30 sec, for 34 cycles, followed by 72 °C for 10 min. Polymerase chain reaction products were visualized by ethidium bromide staining after agarose gel electrophoresis.

**Immunohistochemical Methods**
In addition to H&E light microscopy examination, 4-µm tissue sections were cut from paraffin blocks and mounted on electrically charged glass slides. The sections were heated in an oven at 60°C for 45 min, deparaffinized in three changes of xylene solution, and dehydrated in decreasing alcohol grades for 5 min each. Endogenous peroxidase was quenched by immersion in 3% hydrogen peroxide for 30 min. An antigen retrieval method followed, using a microwave at 140 joules and antigen retrieval solution (BioGenex, San Ramon, CA) for 30-min periods. The sections were incubated overnight at 4°C in moisture chambers with a battery of MAbs including anti-IL-6, anti-COX2 (Transduction Laboratories, Lexington, KY), and anti-VEGF-C (Dako Corp., Santa Barbara, CA) at dilutions of 1:50, 1:25, and 1:50, respectively. Antibody binding was detected by subsequent incubation with a biotinylated secondary antibody and streptavidin peroxidase complex (ABC kit; Vector Labs, Burlingame, CA). Chromogenic development was obtained by the immersion of sections in a 3,3’-diaminobenzidine solution (0.25 mg/ml with 3% hydrogen peroxide). The slides were counterstained with Mayer’s hematoxylin (Biogenex) and coverslipped after the application of mounting medium. The results for IL-6, COX2, and VEGF-C MAbs were reported as a combined score of distribution and intensity as described in previous work on p53 (50). For the purpose of statistical analysis for anti-IL-6, anti-COX2, and anti-VEGF-C, a score of 0 or 1 was considered baseline or normal expression, whereas scores of 2 or more were considered overexpression.

**Statistical Analysis**
Statistical analysis were performed using SPSS version 10 (Chicago, IL). The Spearman rank-order correlation coefficient was used to assess the relation among VEGF-C, IL-6, COX2 (using the combined score of intensity and distribution), and other continuous variables. Associations among a variety of variables, including gender, tumor histology, smoking history, and family history of malignancy, were evaluated using the X² test for heterogeneity or Fisher’s exact test as appropriate. Kaplan-Meier analysis was used to assess the relation of disease-free survival to overexpression using the log-rank test. Student’s t test was used to compare the continuous variables including age at diagnosis and lifetime smoking dose expressed in pack-years, by the nominally classified VEGF-C, IL-6, and COX2. Associations were considered statistically significant if the two-tailed P was <0.05.

**Results**

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