

ORIGINAL ARTICLE

The expression and significance of tumor associated macrophages and CXCR4 in non-small cell lung cancer

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Summary

Purpose: The purpose of this investigation was to determine the expression and significance of tumor associated macrophages (TAMs) and CXCR4 in non-small cell lung cancer (NSCLC).

Methods: Immunohistochemical staining was used to analyze the expression of CD68 (TAM surface marker) and CXCR4 in 68 cases of NSCLC and 17 cases of normal lung tissue.

Results and Conclusion: The positive rate of CD68 was 66.2% (45/68) and CXCR4 was 61.8% (42/68) in the lung cancer tissues, while the rates in normal tissues were statistically significantly lower at 27.3% (3/17) and 11.8% (2/17),

respectively. The tissue expressions of CD68 and CXCR4 in NSCLC tissues were significantly correlated with a higher TNM staging and lymph node metastasis ($p < 0.05$); and the expression of CD68 was positively correlated with the expression of CXCR4 ($r = 0.461$, $p = 0.000$). The TAMs infiltration and the expression of CXCR4 were closely correlated with the cellular carcinogenesis and development of NSCLC and their combined detection could provide a potential target for clinical diagnosis and adjuvant therapy for patient with NSCLC.

Key words: CD68, CXCR, immunohistochemistry, NSCLC, tumor associated macrophage

Introduction

Non-small cell lung cancer (NSCLC) is a malignant tumor with morbidity and mortality rates ranking highest of all tumors worldwide [1]. NSCLC will characteristically metastasize to local and distant lymph nodes and the 5-year survival rate of patients with distant metastasis is less than 5% [2]. The causes of tumorigenesis are multifactorial; epidemiological studies have reported that approximately 25% of all cancers are caused by chronic inflammation which is mediated by inflammatory factors and cells. Tumor associated macrophages (TAMs) are inflammatory cells that invade into the tumor interstitium and play an important role in

inflammation and the process of tumor development [3]. The tumor inflammatory microenvironment favors tumor metastasis by regulating the transformation of epithelial to mesenchymal cells [4]. Chemokines and their receptors also play an important role for the invasion and metastasis of tumor cells. CXC chemokine receptor 4 (CXCR4), one of the chemokine receptors in CXC receptor family, plays a significant role in NSCLC invasion [5]. CXCR4, a member of G protein-coupled receptor (GPCR) family, is the main co-receptor of human immunodeficiency virus type 1 and is primarily involved in signal transduction.

The interaction and mechanism of the infiltration related to TAMs and CXCR4 in NSCLC is not well understood. The purpose of this investigation was to determine the expression of CD68 and CXCR4 protein in NSCLC tissues and to determine whether there is any correlation between the expression of CD68 and CXCR4 in NSCLC.

Methods

General disease information

Paraffin-embedded lung tissue samples from 68 NSCLC patients (50 male and 18 female) and 17 healthy individuals (12 male and 5 female) archived from January 2015 to December 2016 at the Department of Pathology of Hebei General Hospital were selected. The median age was 61 years (range 45-73) in the normal group and 65 years (range 48-79) in the NSCLC group. According to pathological diagnosis, there were 32 cases of adenocarcinoma, 26 cases of squamous cell carcinoma, and 10 cases of other types of cancer; 17 cases were highly differentiated, 30 cases moderately differentiated, and 21 cases poorly differentiated. Forty-three cases had lymph node metastasis and 25 cases had not. According to TNM pathological staging (UICC, 2009), 9 cases had stage I, 20 cases stage II, 24 cases stage III and 15 cases stage IV. No patient was treated with radiotherapy, chemotherapy or immunotherapy before operation and all had complete clinicopathological materials. The study was approved by the ethics committee of Hebei General Hospital and informed consents were signed by the patients and/or guardians.

Immunohistochemistry

Immunohistochemistry was performed using the streptavidin-peroxidase method. Tissue sections were dewaxed to water to expose antigens. Rabbit anti-human CXCR4 monoclonal antibody was purchased from Abcam Company, UK and mouse anti-human CD68 monoclonal antibody, S-P kits, and DAB developing kits were all purchased from Beijing Zhong Shan Gold Bridge Biotech Company. Three percent H₂O₂ was added to tissue sections for 15 min at room temperature. The primary antibody was added and the sections maintained in the kit overnight at 4°C. After phosphate-buffered saline (PBS) washing, the secondary horseradish peroxidase conjugated antibody was added and the sections were incubated at room temperature for 30 min. 3,3'-Diaminobenzidine (DAB) developing was conducted after PBS washing. The sections were then subjected to hematoxylin staining, dehydration and transparentizing,

followed by neutral resin glue for mounting and observation. Positive controls were performed using known positive tissue sections, and negative controls were performed using PBS to replace the primary antibody. All sections were read using a double-blinded method. CD 68 positive cells were characterized by brown-yellow coarse particles in the cytoplasm, and CXCR4 positive cells were characterized by brown-yellow granules in the cytoplasm. Scoring according to staining intensity was as follows: 0 for no color, 1 for light yellow, 2 for brown-yellow and 3 for sepia. Scoring according to the percentage accounted by positive cells was as follows: 0 for negativity, 1 for <5% positive cells, 2 for 6-50% positive cells, 3 for 51- 75% positive cells and 4 for >75% positive cells. If the multiplied value of the two scores was >3, the tissue was considered as having a positive expression.

Statistics

SPSS 13.0 software was used for statistical analyses of the data. Chi-square test was used for comparison of pair-wise rate; correlation analysis used the Spearman rank correlation analysis and $p < 0.05$ was considered statistically significant.

Results

The positive rate of CD68 was 66.2% (45/68) and of CXCR4 was 61.8% (42/68) in the lung cancer tissues, while the rates in normal tissues were statistically significantly lower at 27.3% (3/17) and 11.8% (2/17), respectively (Table 1). The expressions of either CD68 or CXCR4 showed no correlation to patient gender or age, but were positively correlated with TNM staging and lymph node metastasis. Both antigens showed a statistically significantly (for CD68: $\chi^2=18.550$, $p=0.000$; for CXCR4: $\chi^2=14.058$, $p=0.003$) higher positive rate in NSCLC tissues along with the increasing of TNM stage. More undifferentiated NSCLC tissues were positively correlated ($p=0.023$) with a higher expression rate of CXCR4. The positive expression rate of CXCR4 in adenocarcinoma was 78.1%, significantly higher ($p=0.014$) than that of squamous cell carcinoma and other tumor types (Table 2).

Spearman rank correlation analysis showed that the expression of CD68 was positively correlated with the expression of CXCR4 ($r=0.461$, $p=0.000$, Table 3).

Table 1. Expression of CD68 and CXCR4 protein in normal lung and NSCLC tissues

Lung tissue	n	CD68		χ^2	p value	CXCR4		χ^2	p value
		+	-			+	-		
Normal lung tissue	17	3	14			2	15		
NSCLC tissue	68	45	23	13.030	0.000	42	26	13.617	0.000

Table 2. Correlation between expressions of CD68 and CXCR4 in NSCLC tissues and clinicopathological features

Features	n	CD68		x ²	p value	CXCR4		x ²	p value
		+	-			+	-		
Age, years									
≤60	23	14	9			12	11		
>60	45	31	14	0.473	0.508	30	15	1.354	0.245
Gender									
Male	50	35	15	1.234	0.267	34	16		
Female	18	10	8			8	10	3.110	0.078
Tissue type									
Adenocarcinoma	32	21	11			25	7		
Squamous cell carcinoma	26	18	8	0.283	0.868	14	12	8.590	0.014
Other types	10	6	4			3	7		
Differentiation grade									
High	17	11	6			8	9		
Moderate	30	21	9	0.384	0.825	16	14	7.560	0.023
Low	21	13	8			18	3		
TNM stage									
I	9	3	6			2	7		
II	20	8	12			9	11		
III	24	20	4			18	6		
IV	15	14	1	18.550	0.000	13	2	14.058	0.003
Lymph node metastasis									
Yes	43	36	7						
No	25	9	14	13.734	0.000	10	15	7.930	0.005

Table 3. Correlation between the expression of CD68 and CXCR4 proteins in NSCLC tissues

CXCR4	CD68		Total	r	p value
	+	-			
+	35	7	42	0.461	0.000
-	10	16	26		
Total	45	23	68		

Discussion

Carcinogenesis is regulated by a multitude of factors including the internal environment of the tumor cells (i.e. the tumor microenvironment). The tumor microenvironment is comprised of proliferating tumor cells, tumor stroma, blood vessels, infiltrating inflammatory cells, and a variety of associated tissue cells that can be broadly divided into three major categories: the immune, inflammatory and hypoxic microenvironment. TAMs are an important part of the tumor microenvironment and are involved in carcinogenesis, accounting for approximately 50% of the cells of the solid tumor

[6]. Ke et al. [7] reported that, via the toll-like receptors (TLRs) signal pathway, TAMs can closely link the inflammatory stimulus with the proliferation of tumor cells in NSCLC and lead to tumor growth and metastasis. The results of this investigation have shown a positive and statistically significantly higher than normal expression of CD68 positive cells in NSCLC tissues, indicating not only a sizeable number of macrophages in the inflammatory infiltration of the tumor, but also suggesting these cells are important mediators of the tumor inflammatory microenvironment. Yuan et al. [8] have reported a high presence of CD68 positive cells in many solid tumors, Tewari et al. in breast cancer [9], Zhang et al. in gastric cancer [10], and Badawi et al. in colon cancer [11], and have postulated that they play a key role in tumor infiltration, invasion, and metastasis. The results of this investigation also showed high expression of CD68 positive cells that was positively correlated with a higher TNM stage (worse stage) and lymph node metastasis, indicating that the TAMs in the microenvironment of NSCLC may be involved in the tumorigenesis of NSCLC.

The tumor inflammatory microenvironment favors the chemotaxis and tumor transfer ability,

which involves chemokines and their receptors. Tumor cells can secrete certain chemokine(s) and its (their) receptor(s) and their interaction can promote the proliferation of cancer cells, prevent their apoptosis and indirectly modulate tumor growth by inducing factors that promote angiogenesis [12]. The chemokine receptors that appear to have important roles in tumors are CCR7 and CXCR4. Chemokine CXCL12 (also known as stromal cell derived factor 1 (SDF-1) is the specific ligand of CXCR4 and the interaction between CXCL12 and CXCR4 mediates signal transmission among cells involved in the regulation of the immune system that influences tumor cellular growth. The results of this investigation showed that the positive expression of CXCR4 in NSCLS tissues was statistically higher than in normal lung tissues, indicating that CXCR4 is closely correlated with the tumorigenesis of NSCLC. Lefort et al. and Chen et al. [13,14] reported a very low to absent expression of CXCR4 in normal breast tissues while it was highly expressed in breast cancer tissues. In this investigation, the positive expression of CXCR4 in adenocarcinoma was significantly higher ($p=0.014$) compared with squamous cell carcinoma and other tumor types. Sterlacci et al. [15] showed that CXCR4 is often expressed in squamous carcinoma, however there can be regional differences within the tumor and a large sample size may be required for detection. The results of this research have also shown the expression of CXCR4 is correlated with the grade of differentiation and TNM staging of NSCLC. The expression of CXCR4 in the lymph node metastasis group was statistically higher compared with the group without lymph node

metastasis. These findings agree with the report of Wang et al. [16]. It is speculated that tumor cells with highly expressed CXCR4 tend to be attracted by CXCL12, and through that interaction, the tumor cells will move to a specific transfer site.

TAMs and CXCR4 both play important roles in the tumorigenesis of NSCLC, and CXCR4 is highly expressed in NSCLC tissues with highly expressed TAMs ($r=0.461$, $p=0.000$). It is speculated that there may be interaction between TAMs and CXCR4. The mechanism of action may be a synergistic effect between VEGF and CXCR4 caused by TAMs that may attract mesenchymal derived stem cells (MDSCs) and therefore promote the differentiation of TAMs; a circuit reaction can occur that promotes tumor immunosuppression and tumor growth. TAMs, through pro-inflammatory molecules (e.g. NF- κ B and HIF-1 α), may interact with CXCR4 to promote epithelial-mesenchymal transition and tumor immune escape and play a key role in cellular carcinogenesis, invasion and metastasis of NSCLC. Many growth factors (e.g. EGF, PDGF and TGF- β) secreted by TAMs promote TAMs collection and inhibit tumor immunity, exerting their effect together with CXCR4 and directly or indirectly promoting tumor growth.

To conclude, TAMs and CXCR4 are highly expressed in NSCLC. TAMs and CXCR4 together promote carcinogenesis. Combined detection of TAMs and CXCR4 may provide new targets and novel strategies in the treatment of NSCLC.

Conflict of interests

The authors declare no conflict of interests.

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