Original Article

Study on the Expression Levels of CXCR4, CXCL12, CD44, and CD147 and Their Potential Correlation with Invasive Behaviors of Pituitary Adenomas*

XING Bing#, KONG Yan Guo, YAO Yong, LIAN Wei, WANG Ren Zhi, and REN Zu Yuan

Departments of Neurosurgery, Key Laboratory of Endocrinology, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100730, China

Abstract

Objective To evaluate the factors of CXCR4, CXCL12, CD44, and CD147 as early potential diagnostic biomarkers by determining their expression levels in invasive and non-invasive pituitary adenomas.

Methods Fresh pituitary adenoma specimens were collected from 35 pituitary adenoma (21 invasive and 14 non-invasive) patients who underwent surgical treatment in our Neurosurgery Department between January and April of 2009. The expression levels of CXCR4, CXCL12, CD44, and CD147 were evaluated firstly by flow cytometry, fluorescence microscopy in single cell suspensions, and then by immunohistochemical staining of paraffin tissue sections.

Results Flow cytometric analyses showed that the percentage of CXCR4- and CXCL12-positive cells from invasive pituitary adenomas (IPA) was significantly higher in the single cell suspensions than that from non-invasive pituitary adenomas (nIPA) (P<0.05). Immunohistochemical staining revealed that CXCR4 and CXCL12 staining index scores of the invasive pituitary adenomas were significantly higher than those of the non-invasive pituitary adenomas (P<0.05). In contrast, neither flow cytometry nor immunohistochemical staining demonstrated significant difference between CD44 and CD147 expression levels, respectively.

Conclusion Expression levels of CXCR4 and CXCL12 are correlated with the invasiveness of pituitary adenomas. Therefore, rather than CD44 and CD147, CXCR4 and CXCL12 may potentially serve as biomarkers for early detection of pituitary adenomas.

Key words: Pituitary adenoma; Invasiveness; Flow cytometry; Immunohistochemistry; CXCR4, CXCL12, CD44, and CD147

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INTRODUCTION

Pituitary adenoma is one of the most common tumors in the central nervous system (CNS), and its incidence has a tendency to rise. Pituitary adenomas are classified into two categories based on the ability to invade the surrounding tissue, invasive and non-invasive[1]. It is difficult to distinguish these two categories via conventional histopathological examinations, for the difference in cell morphology between them is insignificantly distinct. In the field of neurosurgery, accurate diagnostic and successful treatment of IPA still represents a challenge. However, biological markers can be identified for early diagnosis, and postoperative adjuvant therapeutic strategies can be developed if the molecular mechanisms of IPAs are fully clarified.

Numerous studies have reported that CD44 adhesion molecules are closely related to cell invasion and metastasis of different types of cancers, such as colorectal cancer, gastric cancer, lung cancer,

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#Corresponding should be addressed to: XING Bing, M.D, E-mail: bingxing2@sina.cn; Tel: 13718696377.

Biographical note of the first author: XING Bing, male, born in 1967, M.D, chief physician. Majoring in clinical and basic research on pituitary disease and other seller region tumor.

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breast cancer, and malignant tumors in the urinary and reproductive system[2-8]. In some other tumors, the increasing CD147 expression level means tumors' severe malignancy and invasiveness[9-11]. Numerous chemokines including CXCL12 and CXCR4 and their receptors have been proven to play a role in regulating and controlling tumor growth, angiogenesis, invasiveness, and metastasis which are reflected in breast cancer, ovarian cancer and several other tumors[12-16]. Up to now, few studies have focused on CD44, CD147, CXCL12, and CXCR4 in pituitary adenoma[17-20], which are associated with tumor cells' adhesion and invasion ability, and worthy of further study.

The expression levels of CXCR4, CXCL12, CD44, and CD147 in IPA and nIPA were observed in this study using the flow cytometry technology, immunohistochemical methods and fluorescence microscope observation, and their correlation with the invasiveness of pituitary adenoma was analyzed.

MATERIALS AND METHODS

Patients and Specimen Collection

A total of 35 patients who underwent pituitary adenoma resection in the Department of Neurosurgery of Peking Union Medical College Hospital between January and April of 2009 were recruited, of whom 21 patients were diagnosed as IPA and 14 patients diagnosed as nIPA. The following IPA diagnostic criteria were adopted: 1) Knosp classification grade III-IV tumors and Hardy classification invasive adenomas; 2) Tumor cells confirmed via pathology as invading sellar bone or adjacent dura mater; 3) Tumor cells invading the sphenoid sinus cavity or peripheral vascular and nerve.

Materials

Mouse anti-human CD147 monoclonal antibody and rabbit anti-human CD44 monoclonal antibody were obtained from Abcam Company, USA. Mouse anti-human CXCR4 monoclonal antibody and mouse anti-human CXCL12 monoclonal antibody were purchased from R&D Company, USA. DMIL-PH2 inverted phase contrast microscope and image system (the Leica Company, German), flow cytometry (EPICS XL, Beckman Coulter Company) and DAB staining kit were used.

Methods Immunohistochemical Staining

1) Pathological paraffin blocks of pituitary adenoma operative specimen were cut into continuous sections with 5 μmol/L respectively; then the process as the sequence-deparaffinization, dehydration, and antigen retrieval were followed. 2) The processed sections were incubated with CXCR4, CXCL12, CD44, and CD147 respectively at 4 °C overnight (with the titers of the 4 types of antibodies at 1: 50). 3) Subsequently the sections were washed with PBS for 2 min each time for 3 times, and then the 2nd antibody was added by drop and incubated at 37 °C in water bath for 2 h, and then washed with PBS for 2 min each time for 3 times. 4) DAB solution was used to flush these sections, and then the sections were counterstained with hematoxylin, washed with water, dehydrated and hyalinized. 5) The sections were finally mounted on slides and observed under microscope. The result of immunohistochemical staining is an outcome of comprehensive evaluation which was scored by the pathologist and authors separately to minimize the bias. The result was expressed as the staining index scores which were calculated as the product A and B. A was the categorical scores displaying different proportions in positive stained cells within five random microscopic fields: 1=positive cells <1/3; 2=positive cells between 1/3 and 2/3; and 3=positive cells >2/3. B was the categorical scores representing the staining intensity: 0=no positive staining; 1=light yellow staining; 2=pale brown staining; 3=bright brown staining. Each section was observed under the x 400 microscope, continuous 5 high power fields and 100 cells were observed respectively, mean value was adopted. Because the results are ordinal variable data, the Kruskal-Wallis test was used to analyze them by SPSS 17.0 statistical software. $P<0.05$ is considered statistically significant.

Flow Cytometry Test

Single pituitary adenoma cell suspension was prepared with operative specimen. 20 μL labeled antibodies were added into 100 μL to prepare single cell suspension with CXCR4, CD44, and CD147, respectively, and were then incubated for 20 min at room temperature in dark place and jiggled gently. Afterwards, 2 mL red cell lysis solution was added, and then incubated for 10 min at room temperature in dark place. The solution was centrifuged at 1200 r/m for 5 min, the supernatant was discarded and 2 mL PBS was added. Finally, the solution was centrifuged at 1200 r/m for 5 min, the supernatant was discarded and 500 uL PBS was added to carry out the flow cytometric test within 4 h. Moreover, when samples were prepared for flow cytometric analysis, a slide smear was made for each sample by

dropping1-2 drops of the fluorescent dye-labeled cell suspension on a glass slide. Fluorescence signals were assessed and digital images of the cells were captured under a fluorescence microscope with imaging system. The fluorescent antibody labeled cells can be observed while no-labeled cells are unobservable. The flow cytometry result was recorded as the percentage to show the ratio of fluorescent antibody labeled cells to the tumor cells. Student-*t* test was used for statistic analysis as the flow cytometric data are deemed as continuous variables. $P<0.05$ is considered statistically significant.

RESULTS

Immunohistochemical Findings (Figure 1, Figure 2)

The exact probability of bilateral inspection for CXCR4 and CXCL12 was 0.004 and 0.001 respectively, so the expression levels of CXCR4 and CXCL12 in IPA and nIPA groups were thought to be significantly different. The mean rank of CXCR4 in IPA group was 22.0 which was greater than 12.0 in nIPA group. It was the same with the mean rank of CXCL12, which was 22.64 and 11.04 in each group. The expression levels

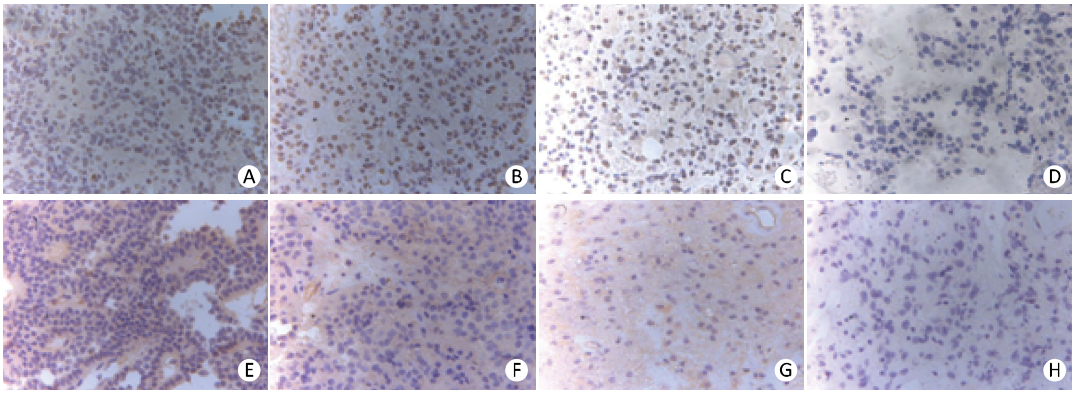

Figure 1. The immunohistochemical staining results of CXCR4 and CXCL12 in pituitary adenoma (×400). A: CXCR4 positive expression in invasive pituitary adenoma, B: CXCR4 positive expression in non-invasive pituitary adenoma, C: CXCR4 weak positive expression in normal pituitary, D: Control, E: CXCL12 positive expression in invasive pituitary adenoma, F: CXCL12 positive expression in non-invasive pituitary adenoma, G: CXCL12 weak positive expression in normal pituitary, H: Control.

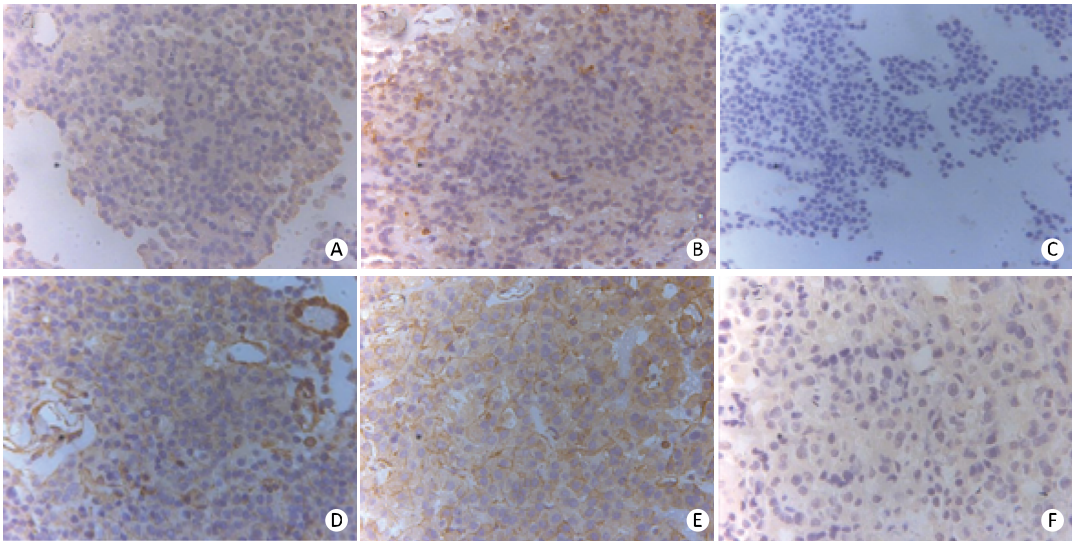

Figure 2. The immunohistochemical staining results of CD44 and CD147 in pituitary adenoma (×400). A: CD44 positive expression in invasive pituitary adenoma, B: CD44 positive expression in non-invasive pituitary adenoma, C: Control, D: CD147 positive expression in invasive pituitary adenoma, E: CD147 positive expression in non-invasive pituitary adenoma, F: Control.

of CXCR4 and CXCL12 in normal pituitary were weak positive and significantly lower than those in the nIPA group. The exact probability of bilateral inspection for CD44 and CD147 was 0.516 and 0.855 respectively, so as we thought, the expression levels of CD44 and CD147 in IPA and nIPA groups had no significant difference. The Spearman (r=0.371, P=0.028<0.05) and Kendall methods (r=0.304, P=0.037<0.05) of linear correlation analysis showed that CXCL12 and CXCR4 in pituitary adenoma had positive correlation.

Findings of Flow Cytometrictest (Figure 3, Figure 4, Table 1, and Table 2)

The t-test showed that there was a significant difference in the expression levels of CXCR4 between the IPA and nIPA groups (t=3.406, P=0.002<0.05), but no significant difference was seen for CD44 (t=1.248, P=0.221>0.05) and CD147 (t=1.776, P=0.085>0.05) between these two groups. The cells were marked by CXCR4, CXCL12, CD44, and CD147 under fluorescence microscope.

DISCUSSION

Pituitary adenoma is a common intracranial benign tumor, causing a series of neuroendocrine disorders and severe dysfunctions. The diagnosis and treatment for pituitary adenoma have achieved significant improvement with recent scientific and technological advances. However, how to early diagnose and effectively treat this adenoma remains a major challenge to worldwide neurosurgeons. Currently, the rate of surgical resection is low, the operation risk is high, and the mortality rate and recurrence rate also remain high. It is therefore urgent to develop a novel strategy for early diagnosis and management.

CXCL12 and CXCR4 can modulate the expression and function of cell surface integrin, thereby promoting the tumor adhesion[21-24]. Cardones[25] found that CXCL12 and CXCR4 promoted tumor metastasis *in vivo* and *in vitro* experiments. Cell adhesion molecules play important roles in the process of the tumor invasion and metastasis[26-29]. It

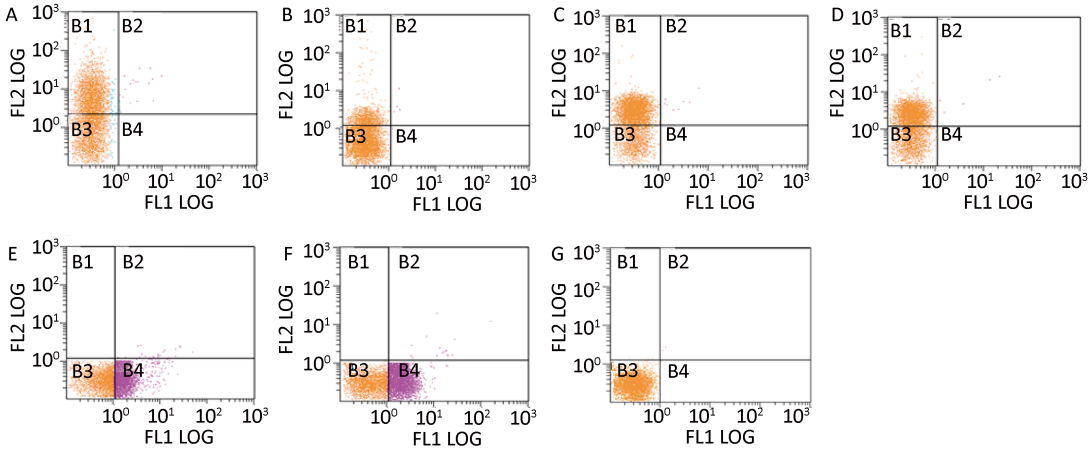

Figure 3. The results of flow cytometric test of CXCR4, CD44, and CD147 in pituitary adenoma. A: CXCR4 in invasive pituitary adenoma, B: CXCR4 in non-invasive pituitary adenoma, C: CD44 in invasive pituitary adenoma, D: CD44 in non-invasive pituitary adenoma, E: CD147 in invasive pituitary adenoma, F: CD147 in non-invasive pituitary adenoma, G: Control.

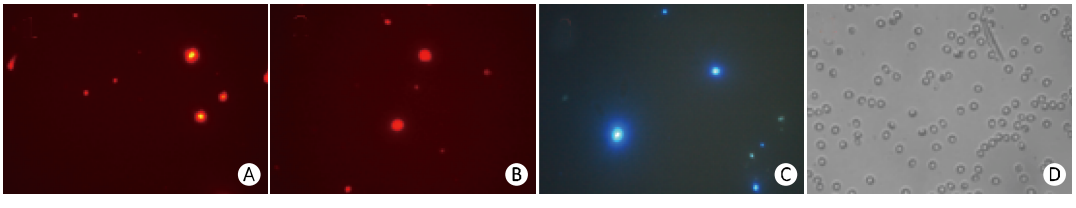

Figure 4. The fluorescence staining results of CXCR4, CD44, and CD147 in pituitary adenoma (×400). A: CXCR4-labeled cells, B: CD44-labeled cells, C: CD147-labeled cells, D: Control.

Table 1. The Flow Cytometry Results of IPA

No.	Sex	Age	Function	Location	CXCR4(%)	CD44(%)	CD147(%)
1	Female	26	PRL	sella floor	64.75	34.80	65.23
2	Female	75	NF	sella floor	25.43	12.38	25.12
3	Male	35	GH	sella floor	44.81	41.60	52.07
4	Male	65	NF	sella floor	75.47	63.38	72.03
5	Male	43	PRL	sella floor	68.72	48.03	81.57
6	Female	57	GH	sella floor	76.48	41.64	37.37
7	Female	70	NF	Cavernous sinus	1.59	35.86	15.96
8	Male	39	NF	Cavernous sinus	44.84	21.85	18.17
9	Female	54	NF	Cavernous sinus	31.05	23.98	49.25
10	Male	39	NF	Cavernous sinus	34.02	17.27	32.58
11	Male	44	PRL	Cavernous sinus	15.42	4.96	25.64
12	Female	25	NF	Cavernous sinus	49.40	13.67	60.68
13	Male	58	NF	Cavernous sinus	23.27	17.37	8.23
14	Male	64	NF	Cavernous sinus	11.57	83.80	50.47
15	Female	46	NF	Cavernous sinus	30.21	77.56	52.43
16	Female	41	GH	Cavernous sinus	30.16	59.65	54.78
17	Male	59	NF	Cavernous sinus	34.31	73.15	52.68
18	Male	63	NF	Cavernous sinus	1.13	91.92	28.52
19	Male	55	GH	Cavernous sinus	20.42	67.03	34.40
20	Female	32	PRL	Cavernous sinus	17.90	91.28	37.13
21	Male	28	PRL	Cavernous sinus	8.54	93.36	40.99

Table 2. The Flow Cytometry Results of nIPA

No.	Sex	Age	Function	CXCR4(%)	CD44(%)	CD147(%)
1	Male	26	GH	14.14	2.84	2.59
2	Male	56	NF	19.88	10.95	9.72
3	Female	27	GH	0.66	0.10	39.87
4	Male	37	GH	5.21	4.62	34.82
5	Male	53	GH	5.77	15.00	12.18
6	Male	67	NF	18.12	40.35	32.87
7	Female	45	NF	10.43	62.12	30.07
8	Female	53	ACTH	17.37	34.33	38.48
9	Male	48	GH	23.37	53.15	44.27
10	Female	44	NF	27.67	59.25	41.20
11	Male	47	GH	12.05	54.82	27.52
12	Female	71	NF	28.44	63.74	47.14
13	Female	35	ACTH	6.72	45.18	36.55
14	Male	46	GH	21.07	63.43	50.33

was assumed that the standard isoform of CD44 (CD44s) and variant CD44 (CD44v) played different respective roles in tumor, since CD44 proved to be a member of the cell adhesion molecule family. CD44s is a risk factor to the tumor growth , while CD44v is a risk factor to the tumor metastasis[30].

In this study, we attempted to investigate whether the expression level of CXCR4, CXCL12, CD44, and CD147 were correlated with the pituitary adenomas progression. We evaluated the expression of CXCR4, CXCL12, CD44, and CD147 first qualitatively with immunohistochemical staining and next quantitatively with flow cytometry. Immunohistochemical methods mainly reflect the expressing location of markers in tumor cells. Although we can acquire the expression strength of the markers, the bias is evident. Flow cytometry is a semi-quantitative method, which can show the ratio of expressive marker cancer cells in the tumor cells, so it is a more objective and accurate method. The expressive cells with fluorescent markers can be visually observed under fluorescence microscope[31]. A comprehensive judgment was made by adopting Knosp and Hardy classification method combining with intraoperative and pathological findings[32-33]. Results from both immunostaining and flow cytometry analyses demonstrated that there was a positive correlation between the level of CXCR4 and CXCL12 and the progression stage of pituitary adenomas (r=0.371, P<0.05), suggesting that CXCR4 and CXCL12 played a role in the regulation of pituitary adenoma invasiveness.

In contrast, the levels of CD44 and CD147 expression were not significantly different between IPA and nIPA. Nevertheless, this preliminary observation needs to be further confirmed in future studies because it is different from the two other studies[19-20]. Duan[19] found that the expression level of CD44s in IPA was significantly higher than that in nIPA, while the expression level of CD44v was low both in IPA and nIPA. Huo[20] showed that the expression level of CD147 in IPA was significantly higher than that in nIPA. The different results may be caused by adopting different invasive criteria, because Duan used the improved Hardy classification method as invasion criteria, while Huo used the comprehensive judgment standard. In addition, the sample size was not large enough to reflect the statistical difference. Although the domestic and foreign scholars thought that CD44 and CD147 had certain significance in malignant tumors' invasion, the pituitary adenoma is a benign tumor, and the malignant degree is not high enough, which may not play a key role in the process of pituitary adenomas invading.

In summary, a significant higher expression of CXCR4 and CXCL12 was detected in IPA than in nIPA. In contrast, there was no significant difference in the expressions of CD44 and CD147 between IPA and nIPA. These observations suggest that CXCR4 and CXCL12 may be correlated with the invasiveness of pituitary adenoma and thereby may potentially serve as a new marker for early detection of malignant pituitary adenomas.

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