

Biological characteristics of a specific brain metastatic cell line derived from human lung adenocarcinoma

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Abstract To study the expression of VEGF, MMP-9, EGFR, and S100B in a highly brain metastases sub-clone cell line, PC14/B. The in vitro metastases-related behaviors of PC14/B cells, such as adhesion to extracellular matrix (ECM), migration, and invasion were determined and compared with primary PC14 cells and A549 cells that do not metastasize to brain. The expression of vascular epithelial growth factor (VEGF), matrix metalloproteinase 9 (MMP-9), S100B, and epidermal growth factor receptor (EGFR) in the above three cell lines were measured by immunohistochemical staining and Western blot assay. The PC14/B cells have enhanced abilities of adhesion, migration, and invasion than PC14 cells and A549 cells. The expression levels of VEGF and MMP-9 in PC14/B cells are much higher than in PC14 and A549 cells. Two protein polymers of S100B are expressed specially in PC14/B cells. The expression of EGFR has a significant lower level in PC14 cells than in the other two cell lines. The increased expression of VEGF and MMP-9 may lead to the enhancement of adhesion, migration, and invasion of PC14/B cells. The expression of EGFR in PC14/B cells may have negative correlation with their capacities of metastasizing to brain. The specific expression of S100B in PC14/B cells strongly suggest that S100B might be a potential target for developing new therapy to brain metastases of lung cancer.

Keywords Lung cancer · Metastases · Vascular endothelial growth factors · Matrix metalloproteinase-9 · S100B · Epidermal growth factor receptor

Introduction

Lung cancer is the most common primary tumor leading to brain metastasis. About 30% brain metastases derive from lung cancer [1]. The brain metastasis is a common cause of death in lung cancer patients. Routine brain scans of patients with newly diagnosed non-small-cell lung cancer identify brain metastases in 3–10% of patients. Brain metastases were observed in about 15% of patients with small-cell lung cancer.

The detailed mechanisms of brain metastases are still unclear. Lack of fundamental research for molecular mechanism of brain metastases from lung cancer resulted in hindering treatment for lung cancer with brain metastasis.

Among major theories concerning cancer metastasis, the one of “seed and soil” developed by Stephen Paget was extensively accepted. It was perceived that certain organs must provide appropriate conditions for development of organ-specific metastases [2]. During the past decades, biological studies have identified important molecular interactions between tumor cells (seeds) and stromal environment (soil) that dictate the tissue-specificity of metastasis formation. The discovery of numerous growth factors, chemokines, guidance molecules, signaling pathways, and more importantly, new genes provides a new identity for organ-specific metastasis [2–4].

Vascular epithelial growth factor (VEGF) is a powerful enhancing factor of angiopoiesis in tumor growth and tumor metastasis. VEGF can fuel the permeation of neoplastic cells into vascular endothelial cell (VEC) of brain tissue [5] and promote brain metastases [3, 6].

Matrix metalloproteinase 9 (MMP-9) can degrade the type IV collagen and other compositions of extracellular matrix (ECM). It is considered to have closely correlation

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with metastases and angiogenesis [7–9], which is important in brain metastases of mammary cancer [10].

S100 family, calcium-binding protein of the EF-hand type, is homodimer or heterodimer composed of 2 different subunits (A and B). The variegated recombination of S100 genes in human cell can lead to varied malignant tumors, especially the metastases to central nervous system (CNS). The concentration of S100B protein in cerebrospinal fluid (CSF) can be used to determine the degree of brain injury and the body's immune station, evaluating the therapeutic effect and prognosis [11]. It has been proposed that S100B contributed to tumorigenesis by inhibiting the function of the tumor suppressor protein p53 [12, 13], and to regulate cell proliferation and differentiation by stimulating the activity of the mitogenic kinases Akt [14] and Ndr (PKB) [15].

Epidermal growth factor receptor (EGFR) is related with more than 70% malignant tumor [1, 16] and is important in tumor growth, such as angiogenesis, metastasis, adherence, and inhibition of apoptosis [17, 18].

We have established a model of brain metastasis of non-small-cells lung cancer by intravenous inoculation of human lung adenocarcinoma PC14 cells into nude mice [19]. The clones that selectively metastasize to mouse brain (PC14/B) were isolated.

In the present study, we compared the expression of VEGF, MMP-9, EGFR, and S100B in PC14/B (specific metastasize to brain), PC14 (metastasize to brain occasionally), and A549 (never metastasize to brain) cell lines.

Materials and methods

Cell lines, antibody, and reagents

The human adenocarcinoma cell lines, PC14 and A549, were gifts from professor Sone (Tokushima University in Japan). The cell line PC14/B was established in our lab as previously described [19]. Extracellular matrix gel (ECM gel, originated from mouse EHS carcinoma) was from Sigma (St. Louis, USA). Millicell Chamber was from Millipore (New Jersey, USA). Elivision™ plus Kit, mouse anti-human VEGF, MMP-9 monoclonal antibody were from MaiXin-Bio (Fuzhou, China). Mouse anti-human S100 β chain antibody was from Boster (Wuhan, China). Rabbit anti-human EGFR polyclonal antibody was from Bosen (Beijing, China). Mouse anti-human EGFR antibody derived from Zhongshan Goldenbridge (Beijing, China). Mouse anti-human GAPDH monoclonal antibody was from Chemicon (Rosemont, USA). Goat anti-mouse IgG conjugated with horseradish peroxidase was from Pierce (Rockford, USA). Goat anti-rabbit IgG conjugated with horseradish peroxidase was from DingGuo (Beijing,

China). ECL plus kit was from Amersham Bioscience (Piscataway, UK). RIPA lysis buffer was from Beyotime (Nanjing, China).

In vitro adhesion assay

Cellular adhesion to ECM gel was quantified using a crystal violet-based adhesion assay. The 96-well plates were coated with ECM gel, which has been reconstructed to form a continuous thin layer, and 1% heat-treated bovine serum albumin (BSA) was added to block non-specific adhesion. Cells were suspended in Hanks' balanced salt solution (HBSS) containing 1% BSA. 1×10^4 cells were added to each well of an ECM gel-coated microtiter plate and incubated for 1 h at 37°C. The plate was washed 3 times with 100 μ l HBSS to remove unattached cells. Adherent cells were quantified by MTT assay. Cellular adhesion to wells coated with BSA was used as a control for non-specific adhesion. Absorbance at a wavelength of 490 nm was measured to determine the adhesion. Each independent experiment was performed 8 times, with 10 determinations for each condition tested. The percentage of adhesion was calculated as follows: adhesion = (experimental/control)–1.

In vitro migration and invasion assays

Cellular invasiveness was quantified according to the method described previously [20]. A millicell with an 8- μ m porosity cell-permeable polycarbonate filter was covered with ECM gel to form a continuous thin layer, and it was placed on a 24-cell culture plate. 1×10^5 tumor cells of A549, PC14, PC14/B were then added into the millicells, respectively. As a chemoattractant, NIH3T3 fibroblast conditioned medium was used outside of the millicell. Cultivation for 48 h was followed by MTT assay to quantify the number of infiltrating tumor cells. Cellular migration was quantified in the same manner with the exception that ECM gel was omitted. Each independent experiment was performed 4 times, with 8 determinations for each condition tested.

Immunocytochemistry analysis and Western blot analysis

At the end of the culture, period plastic chambers were removed, and slides were rinsed in phosphate buffered saline (pH 7.4), fixed in acetone at 4°C for 5 min, and air dried. Before the immunostaining was performed, the cells on the slides was treated with Triton X-100 (0.3%). Briefly, slides were incubated for 1 h at room temperature with primary antibodies against VEGF, MMP-9, EGFR (mouse anti-human), and S100 β . Solution A and B were added

successively. Following a rinse in PBS, the color reaction was accomplished using the DAB reaction. The slides were mounted with coverslips and examined under microscopy. Negative controls were prepared by using irrelevant isotype matched antibodies in place of the primary antibodies. To further investigate the expression pattern of EGFR and S100B in these cell lines, Western blot analyses were proceeded. Whole cell lysates were made by RIPA lysis buffer according to the instruction, and insoluble material was removed by centrifugation, and protein concentrations of the cell lysates were determined with Coomassie brilliant blue assay. Cellular proteins (20 µg) were separated by SDS-PAGE, and transferred to PVDF membranes (Millipore, Billerica, USA) as previously described [21]. The same antibody against S100β used for immunocytochemistry was used for immunoblotting, whereas the anti EGFR antibody (rabbit anti-human) was used. Membranes were incubated with primary antibodies for 1 h at room temperature, followed by secondary antibody conjugated to horseradish peroxidase. Detection was performed using the ECL plus kit according to the manufacturer's instructions. The detection of GAPDH was performed as the internal control.

Image analysis and quantitation

Immunocytochemical staining images were obtained by BX60 microscope (Olympus, Tokyo, Japan), and analyzed by Image Pro Plus system (Media Cybernetics, Silver Spring, MD). The intensity of staining was determined by counting IOD (integrated optical density) in 10 different fields for each sample. Western blot images were captured and analysed using G: BOX (Syngene, Frederick, MD) with the GeneSnap/GeneTool softwares (Syngene). For quantitative data, each assay was repeated at least three times. Data were presented as the mean ± SEM. Statistical analyses were performed with *t* test and one-way ANOVA. $P < 0.05$ was considered statistically significant.

Results

Adhesion assay

To determine the adhesive capacity for the three cell lines, we analyzed their adhesion behavior in a cell adhesion assay using 96-well plates coated with ECM. The ratios of cell adhesion for PC14/B, PC14, and A549 are 0.24 ± 0.03 , 0.09 ± 0.03 , and 0.12 ± 0.03 ($P = 0.004$), respectively. The adherence ratio of PC14/B is higher than PC14 ($P = 0.001$) and A549 ($P = 0.011$), meanwhile there is no difference between PC14 and A549 cell lines ($P = 0.411$). The D values of the three cell lines in ECM

group are 0.70 ± 0.09 , 0.56 ± 0.08 , and 0.60 ± 0.05 , respectively, which was significant higher in PC14/B than in PC14 ($P = 0.001$) and A549 ($P = 0.007$). These data showed that the adhesive ability of PC14/B cells with the matrix was higher than that of PC14 cells, whereas there was no significant difference between PC14 and A549 in adhesive ability. After incubation of 1 h, the number of PC14/B cells invading the membrane was more than the other two cell lines (Fig. 1).

In vitro migration and invasion assay

The migration ability of the three cell lines were quantified using a Boyden chamber assay. After 12-h incubation, the three cell lines (PC14/B, PC14, and A549) spread through the pores of the filter and into the lower chamber. The values of D determined by MTT assay were 0.51 ± 0.12 , 0.32 ± 0.13 , and 0.19 ± 0.06 ($P = 0.007$), respectively. The migration ability of PC14/B was higher than PC14 ($P = 0.034$) and A549 ($P = 0.002$), whereas there was no significant difference between PC14 and A549 ($P = 0.126$) (Fig. 2a). In a modified Matrigel Boyden chamber assay, the values of D determined by MTT assay are 0.69 ± 0.17 , 0.38 ± 0.06 , and 0.27 ± 0.06 , respectively. The invasive capability of these three cells was significantly different each other ($P = 0.001$). The invasive ability of PC14/B was higher than that of PC14 ($P = 0.005$) and A549 ($P < 0.001$). The invasive capability of PC14 is higher than that of A549 ($P = 0.049$) (Fig. 2b).

Expressions of VEGF, MMP-9, EGFR, and S100β in the three cell lines

To investigate the expression of VEGF, MMP-9, EGFR, and S100β in the three cell lines, immunocytochemical staining was proceeded. The system of Image-Pro Plus 6.0 was used for analyzing the semi-quantity results according to the light intensity of staining. We found that the expressions of VEGF and MMP-9 detected in membrane of PC14/B and A549 cells were much higher than that in PC14 cells. The expression intensity of S100β, which can be detected in membrane and in cytoplasm of PC14/B and PC14 cells gradually weakened, and no staining was seen in A549 cell line. The expression of EGFR observed in membrane and cytoplasm of PC14/B cells was much lower than that of PC14 and A549 cells (Fig. 3; Table 1).

The protein expression of EGFR and S100β in the three cell lines

Western blot was used to further determine the expression of the proteins of EGFR and S100β in the cell lines. We

Fig. 1 The adhesive ability of PC14/B, PC14, and A549 cells investigated by crystal violet-based adhesion assay. PC14/B (a), PC14 (b), and A549 (c) cells were plated to 96-well plates coated with ECM (A) and BSA (B), and the adherent cells were quantified by MTT assay after regular incubation. The samples were photographed using a 40× objective by an Olympus BX60 microscope

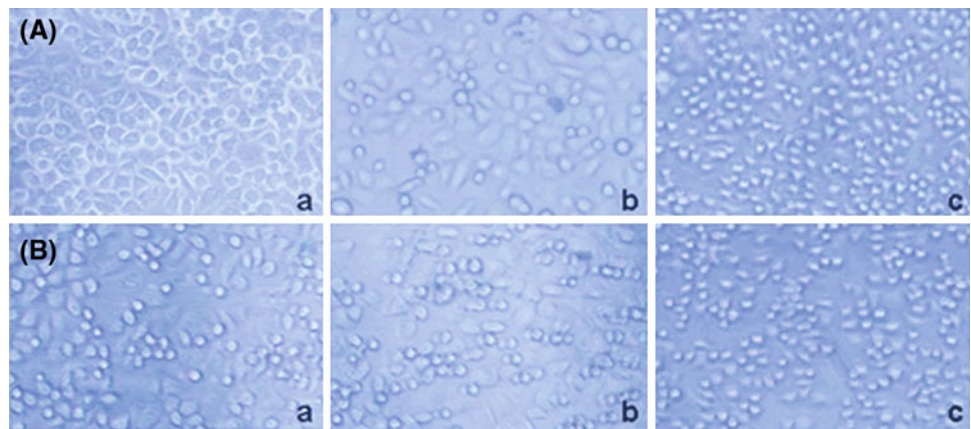
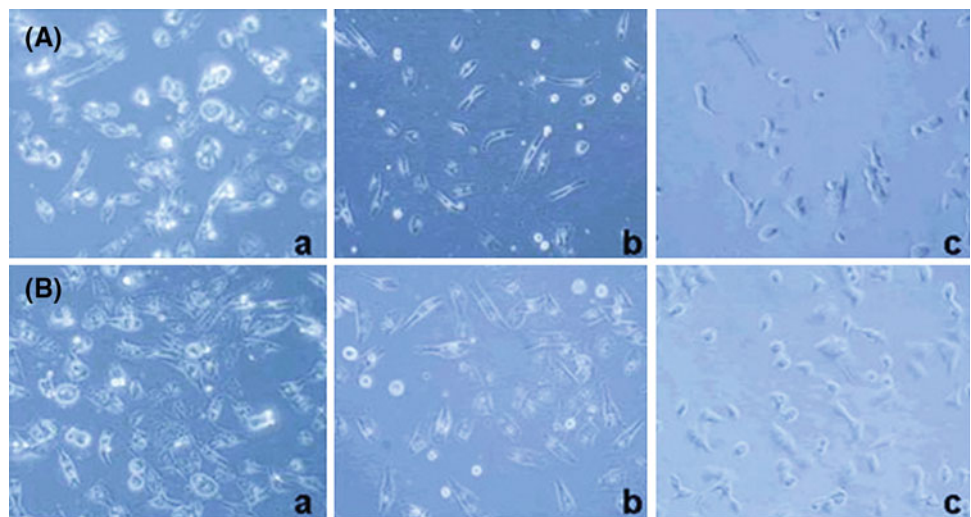


Fig. 2 The migration and invasive ability of PC14/B, PC14, and A549 detected using millicells. The migration (A) and invasive (B) assay of PC14/B (a), PC14 (b), and A549 (c) were performed in Boyden Chamber covered with ECM gel on the filter. Cellular invasion and migration were quantified by MTT assay, with the exception that ECM gel was omitted in the cellular migration assay. The samples were photographed using a 40× objective by an Olympus BX60 microscope



found that the expression of EGFR in PC14/B cells was significantly lower than those in A549 and in PC14 cells. We also found that there were two bands at 32 and 40 kDa, respectively in PC14/B, which can not be observed in either PC14 or A549 cells, and the intensity of bands at 40 kDa was stronger than that at 32 kDa (Fig. 4).

Discussion

Brain metastases are generally believed to result from hematogenous spread of circulating tumor cells or frank tumor emboli [22]. Brain metastases from lung cancer are common, possibly because the pulmonary capillary bed serves as a filter that prevents tumors elsewhere in the body from entering the arterial circulation. About 49.5–60% brain metastases derived from lung cancer. The median survival is measured in months, the percentage of 2-year survivors in single digits and cures rare enough to be the subject of case reports [23]. But the sequence of events that initiates metastatic spread to the brain remains largely unknown. We have established a highly brain metastatic

sub-clone of lung adenocarcinoma named PC14/B originated from PC14 through repeated injecting PC14 cells into nude mice [2]. The in vitro metastases-related behaviors of PC14/B cells were determined and compared with primary PC14 cells and A549 cells, which cannot metastasize to brain.

The classic hypothesis at present is “Seed and Soil Hypothesis” proposed by Paget. As currently conceived, the hypothesis postulates that genetic changes in some cancer cells (the seed) allow them to find the biochemical environment of the brain an especially favorable place where to grow. It has versatile relationships between the microenvironments of organ and tumor cells by a cluster of cytokines [24]. MMP-9 can degrade the type IV collagen and other compositions of extracellular matrix (ECM). It is considered to have closely correlation with metastasis and angiogenesis [7, 9, 10], which is important in brain metastases of mammary cancer [10]. And it can also enhance the activity of angiogenesis of VEGF. Whereas VEGF is a powerful enhancing factor of angiopoiesis in tumor growth and tumor metastasis. VEGF can also promote the secretion of MMP-9 [25]. It has important

Fig. 3 Expression of VEGF, MMP-9, EGFR, S100b in PC14/B, PC14, A549 cells detected by immunohistochemical staining. Cold acetone-fixed PC14/B (a, d, g, j), PC14 (b, e, h, k), A549 (c, f, i, l) cells were incubated with antibody of VEGF (a, b, c), MMP-9 (d, e, f), EGFR (g, h, i), S100 β (j, k, l). The samples were photographed using a 40 \times objective by an Olympus BX60 microscope and analyzed by Image Pro Plus system (Media Cybernetics, Silver Spring, MD). The intensity of staining was determined by counting IOD (integrated optical density) in 10 different fields for each sample

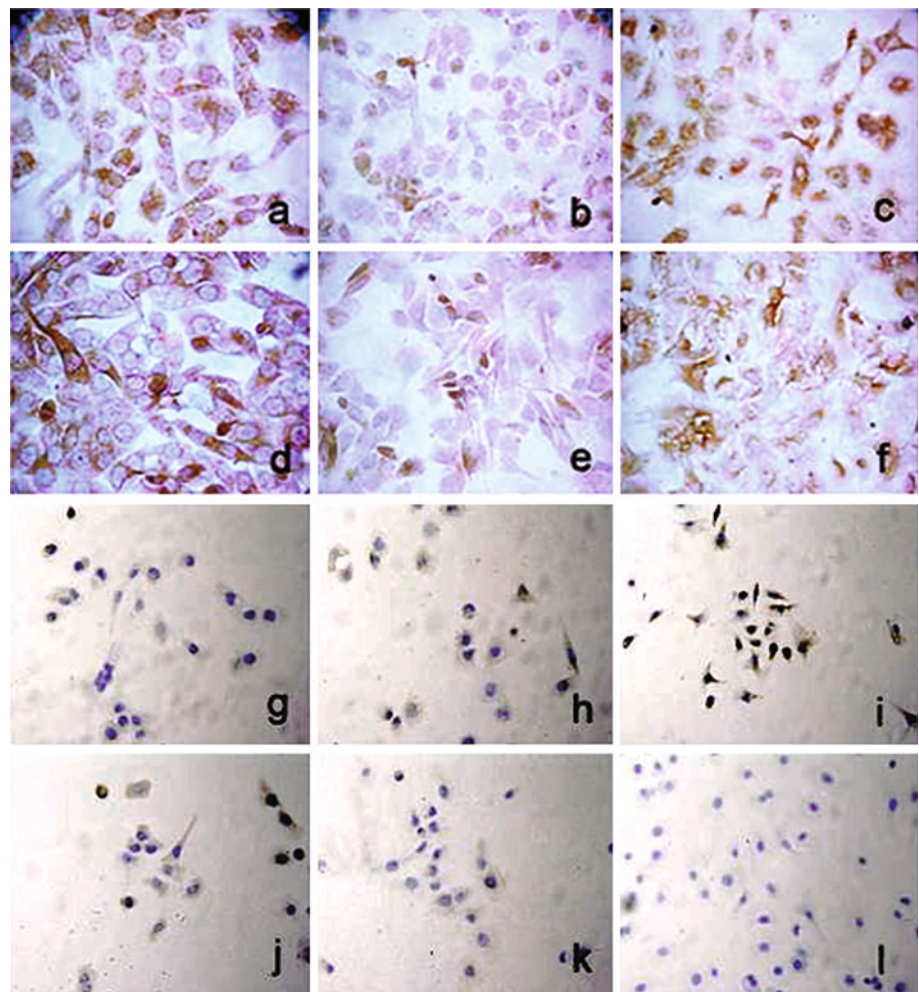


Table 1 General expression of VEGF, MMP-9, EGFR, and S100 β ($n = 10$, $\bar{x} \pm s$)

Cell lines	VEGF (IOD)	MMP-9 (IOD)	S100 β (IOD)	EGFR (IOD)
A549	0.250 \pm 0.019	0.284 \pm 0.015	0.002 \pm 0.001	0.272 \pm 0.006
PC14	0.201 \pm 0.003	0.185 \pm 0.017	0.029 \pm 0.004	0.158 \pm 0.005
PC14/B	0.271 \pm 0.053	0.305 \pm 0.073	0.153 \pm 0.006	0.049 \pm 0.003

mediation in the procedure of neoplastic cells getting through the basal membrane to brain. VEGF can induce lung cancer cells migrating into brain [6] and promote angiopoiesis together with MMP-9.

S100 family, calcium-binding proteins, is usually composed of 2 different subunits (A and B). S-100AB and S-100BB are collectively called S100 β (S100 beta, S100 β). The variegated recombination of S100 genes in human cell involve in varied malignant tumors, especially the metastasis to central nervous system (CNS). In patients with non-small-cell lung cancer, S100 protein in serum is an important biomarker of the integrality of blood brain barrier (BBB) [26]. The determination of S100B protein in cerebrospinal fluid (CSF) is very important in patients with

metastatic carcinoma in CNS, such as determining the degree of brain injury, evaluating the therapeutic effect and prognosis, and so on [11]. S100B can also interact with the p53 protein in a calcium-dependent manner and down-regulates its function as a tumor suppressor, which lead to genesis and metastasis of neoplastic cells [27].

EGFR is biologically active in majority of malignant tumor [1, 16]. EGFR mutations are present frequently in brain metastases [28]. Lapatinib, an inhibitor of EGFR, can partly diminish the brain metastases from mammary gland; Erlotinib, a phosphate tyrosine kinase (PTK) inhibitor, can significantly prolong the life span of patients with non-small-cell lung cancer who suffer from the failure of routine chemotherapy.

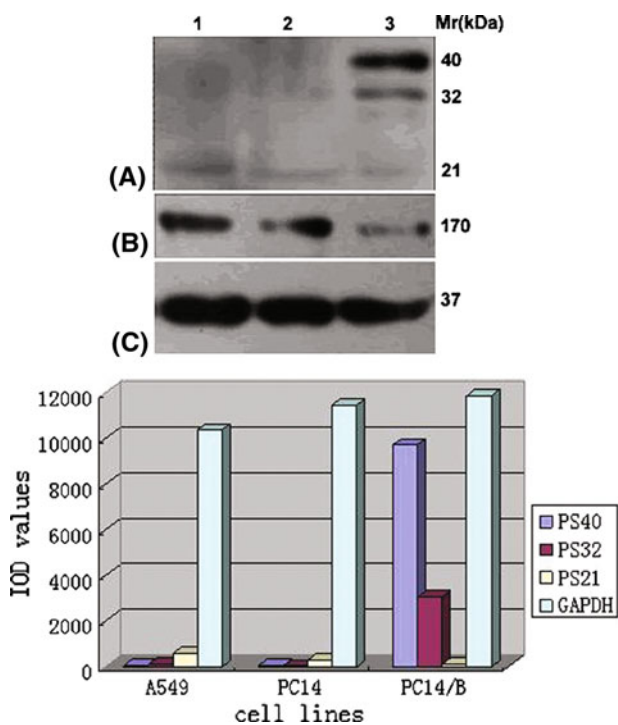


Fig. 4 Expression of S100B and EGFR in PC14/B, PC14, A549 cells detected by Western blot. S100B (A) and EGFR (B) expression were detected under non-reducing 15 and 8% SDS-PAGE, respectively. (C) GAPDH detection was used as internal control. Western blot images were captured and analysed using G: BOX (Syngene) with the GeneSnap/GeneTool softwares (Syngene). The intensity of staining was determined by counting IOD (integrated optical density) obtained from Image Pro Plus system (Media Cybernetics)

The multitude of genotype in different substrain of tumor cells determines their different capacities of metastases and invasion [29]. Only those cell substrains that have special affinity to brain tissue can metastasize to brain [30]. These cell substrains are generated under natural selection by phenotype transformation.

In the present study, we compared the expression of VEGF, MMP-9, EGFR, and S100B in PC14/B (specific metastasize to brain), PC14 (metastasize to brain occasionally), and A549 (never metastasize to brain) cell lines. We have previously screened a highly brain metastatic subclone of lung adenocarcinoma from PC14, which was named PC14/B, and accomplished the evolution artificially. The present results indicated that the PC14/B cells possess significant enhanced abilities of adhesion, migration, and invasion to ECM. The expressions of VEGF and MMP-9 in PC14/B cells are much higher than in PC14 and A549 cells. We conclude that the increased expression of VEGF and MMP-9 may lead to the enhancement of adhesion, migration and invasion of PC14/B cells. We have also found that there are two protein polymers of S100B expressed specially in PC14/B, suggesting that S100B

might be a chemotactic factor in brain metastases of lung cancer. These proteins may take part in destroying the blood brain barrier and interact with neurocyte in brain. In addition, we found that the expression of EGFR in PC14/B is significantly lower than in PC14 and A549. Matsumoto [31] has reported that EGFR mutations present frequently in brain metastases. We, therefore speculate that the expression level of EGFR may have negative correlation with their capacities of metastasizing to brain. Although EGFR inhibitor has been reported to attenuate the progress of lung cancer, the relationship of EGFR and the brain metastases need further study. The specific expression of S100B in PC14/B cells strongly suggest that S100B might be a potential target for developing new therapy to brain metastases of lung cancer.

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