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ORIGINAL ARTICLE

Mechanisms of AEG-1 and CXCR4 gene expression regulating the epithelial-mesenchymal transition pathway involved in brain metastases of breast cancer

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Summary

Purpose: *To study the astrocyte elevated gene (AEG)-1 in breast cancer and the mechanism of the chemokine receptor CXCR4 regulating the epithelial-mesenchymal transition (EMT) involved in brain metastases of breast cancer.*

Methods: *A total of 20 breast cancer patients with and 40 without brain metastases were recruited. The expressions of AEG-1, CXCR4, E-cadherin, N-cadherin and a-SMA were detected by immunohistochemical staining, real-time (RT) quantitative (q) PCR and Western blot respectively in cancer and adjacent normal tissues.*

Results: *The expressions detected in the adjacent normal tissues from both groups showed no significant difference (p>0.05). In the group with brain metastases, AEG-1, CXCR4, N-cadherin, a-SMA, mRNA and the relative expression level of protein were higher than those of patients without brain metastases, while E-cadherin showed the opposite trend.*

Conclusion: *AEG-1 and CXCR4 activate and regulate the EMT pathway to participate in brain metastases.*

Key words: *AEG-1, brain metastases, breast cancer, CXCR4, EMT*

Introduction

The incidence of breast cancer ranks second among all malignant tumors in females, the first being cervical cancer. Comprehensive application of surgery, radiotherapy and chemotherapy, endocrine and targeted therapy can significantly prolong survival of patients [1]. However, the incidence of distant metastases increases at the same time and approximately 30-60% of the patients eventually die of brain metastases [2]. The EMT pathway participates in the local invasion and distant metastases of cancer cells as well as locating the target organ. It therefore plays an important role in the occurrence and development of breast cancer, cervical cancer, gastric cancer and colorectal cancer [3,4]. Its degree of activation is controlled by a variety of genes such as AEG-1 and chemokine receptor CXCR4 [5,6]. As shown by Brown et al. [7], AEG-1 as a proto-oncogene participates in the genesis, progression, invasion and metastases of breast cancer. AEG-1 is found with elevated expression and increased activity in multiple malignant tumors but low to no expression in normal and benign cells. The overexpression of AEG-1 can increase the metastases and invasion of tumors while silencing the gene can lower its malignant biological ability [8], which suggests that AEG-1 may participate in regulating the location of transfer sites. CXCR4 is a member of the G-protein-coupled receptor superfamily and plays an important role in the process of tumor proliferation, infiltration, angiogenesis and metastases and is closely related to the homing and transmis-

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sion of immune cells [9,10]. Therefore, this study focused on the mechanisms of AEG-1 and CXCR4 regulating EMT pathway in brain metastases of breast cancer, providing a new target for early diagnosis and intervention.

Methods

Subject information

A total of 20 patients with brain metastases from breast cancer and 40 breast cancer patients without brain metastases were recruited from June 2014 to June 2016. Breast cancer tissues and adjacent normal tissues (at least 3cm away from the tumor) were obtained. The mean age of patients with brain metastases was 66.6 ± 12.3 years with 5 cases with triple-negative cancer. The mean diameter of the primary tumor was 3.6 ± 0.7 cm. The mean age of patients without brain metastases was 66.5±14.7 years with 11 cases of triple-negative cancer and the mean diameter of primary tumor was 3.7 ± 0.9 cm. The baseline data of two groups were comparable.

Methods

Immunohistochemical staining, RTqPCR and Western blot were used to detect the expressions of AEG-1, CXCR4, E-cadherin, N-cadherin and α-SMA in the tumor tissues and adjacent normal tissues from both groups of patients. Tissue paraffin sections with thickness of about 4μm were made and preserved under -80°C. Three samples were randomly generated using each patient's cancer tissue and adjacent normal tissue.

Immunohistochemical staining

The sections were deparaffinized with xylene and hydrated by gradient ethanol. After incubation for 20 min at 37°C with 3% H_2O_2 normal goat serum was added dropwise and incubated at 27°C for 30 min. Monoclonal primary antibodies of mouse-anti-human AEG-1, CXCR4, E-cadherin, N-cadherin and α-SMA were added dropwise (Jiangsu Biyuntian Technology Co., Ltd, Nangjing, China; working concentration 1:2000). The sections were then incubated in the wet box at 4°C overnight. The normal mouse IgG served as negative control. Then, polyclonal secondary antibodies of rabbit-anti-mouse IgG were added (Beijing Zhongshan-Golden Bridge Biological Technology Co., Ltd, China working concentration 1:500). The sections were incubated at 27°C for 20 min. Enzyme horseradish peroxidase (HRP)-labeled-streptavidin (Sigma, St. Louis, Mo, USA) was then added dropwise and the sections were incubated at 27°C for 20 min. The sections were washed with PBS buffer 5 min for 3 times. After treatments including DAB color development, hematoxylin stain, hydrochloric acid alcohol differentiation, gradient ethanol dehydration and xylene transparentization, the sections were sealed with neutral balsam, dried at room temperature and observed under light microscope. The results were determined using the semi-quantitative method according to the staining intensity and the proportion of stained cells. If the cytoplasm or nucleus were stained to dark brown, it was determined as positive. Zero point meant no positive staining, 1 weak staining, 2 medium staining and 3 strong staining. Positive cells ratio of ≤ 5% was characterized as 0 points, 6-25% as 1 point, 26-50% as 2 points, 51-75% as 3 points and > 75% as 4 points. The two numbers were multiplied with a product of 0-3 categorized as negative and 4-12 categorized as positive.

RT-qPCR

Total RNA was extracted from cells by the conventional Trizol reagent. The concentration and purity were determined by ultraviolet spectrophotometer and cDNA was synthesized using the reverse transcription kit. Shanghai Biological Engineering Co., Ltd. synthesized primers according to the Gene bank sequence (Table 1). The reaction ingredients included 2 μl of cDNA and 3 μl of each of the forward and reverse primers + 0.5μl of Taq polymerase + 1 μl of dNTPs + 3μl of $MgCl_2$ + 10 × 5μl of buffer + 2.5μl of ddH_2O_2. The reaction conditions were 95°C for 5min95°C for 30s, 58°C for 30s and 72°C for 60s for a total of 30 cycles and ended with 72°C for 10min. The 2% agarose gel electrophoresis was used for identification of PCR products. The gel was then analyzed under ultraviolet spectrophotometry

Table 1. Target gene primer sequence

Target gene	*Primer Sequence*	*bp*
AEG-1	5'-AAATAGCCAGCCTATCAAGACTC-3'	325
	5'-TTCAGACTTGGTCTGTGAAGGAG-3'	
CXCR4	5'- GAACTTCCTATGCAAGGCAGTCC-3'	246
	5'- CCATGATGTGCTGAAACTGGAAC-3'	
E-cadherin	5'-ATCAAAGGTATCACGGCAAACG-3'	479
	5'-CGGAGAGCTCGTCCACGTAT-3'	
N-cadherin	5'- GTGCCATTAGCCAAGGGAATTCAGC-3'	337
	5'- GCGTTCCTGTTCCACTCATAGGAG-3'	
α-SMA	5'-CCGAGATCTCACCGACTACCTCA-3'	144
	5'-GCAGTGGCCATCTCATTCTCAA-3'	
GAPDH	5'- CGCGAGAAGATGACCCAGAT-3'	128
	5'-GCACTGTGTTGGCGTACAGG-3'	

with a digital gray line value analysis. The results were recorded using the $2^{-\Delta\Delta Ct}$ method.

Western blot

RIPA lysate was added to the tissue homogenate to extract the total protein of cells, which was quantified using the Coomassie brilliant blue method. The amount of protein in each sample were normalized using a β-actin antibody before each protein assay. Ten μg of total protein were separated by 8% SDS-PAGE. The protein with a negative charge was transferred to the PVDF membrane with the addition of mouse-anti-human AEG-1, CXCR4, E-cadherin, N-cadherin and α-SMA monoclonal antibodies (1:2000, Invitrogen, Carlsbad, California, USA) and left overnight. The rabbit-anti-mouse polyclonal antibodies (1:500, Applied Biosystems, City Foster, California, USA) were then added and incubated at room temperature for 4hrs. The protein was then washed with PBS and applied with electrochemiluminescense (ECL) for protein detection. The results were scanned and analyzed semi-quantitatively using Lab Works 4.5 gel imaging system (Invitrogen, Carlsbad, California, USA) and recorded using Integrated Optical Density (IOD).

Statistics

SPSS20.0 software was used for the statistical analysis. The measurement was expressed as mean ± standard deviation. Independent samples t test was used for inter-group comparisons. The number of count was recorded using the number of cases or their percents. Chi-square test was used for comparisons between groups. A p value less than 0.05 was considered to be statistically significant.

Results

Immunohistochemical staining results analysis

In cancer tissues, the positive expression rates of AEG-1, CXCR4, N-cadherin and α-SMA in patients with brain metastases were significantly higher than in patients without brain metastases ($p<0.05$). There was no significant difference between the two groups in the adjacent normal tissues ($p> 0.05$) (Table 2).

RT-qPCT results analysis

The expressions of AEG-1, CXCR4, N-cadherin and α-SMA mRNA were significantly higher in patients with brain metastases than in patients without brain metastases ($p<0.05$). There was no significant difference between the two groups in the adjacent normal tissues ($p>0.05$) (Table 3).

Western blot results analysis

The expressions of AEG-1, CXCR4, N-cadherin and α-SMA mRNA were significantly higher in patients with brain metastases than in patients without brain metastases ($p<0.05$). There was no significant difference between the two groups in the adjacent normal tissues ($p>0.05$) (Table 4).

Table 2. Immunohistochemical staining analysis

Group	*Cases, n*	*AEG-1* n (%)	*CXCR4* n (%)	*N-cadherin* n (%)	*a-SMA* n (%)	*E-cadherin* n (%)
Cancer tissues with brain metastases	60	31 (51.7)*	33 (55.0)*	28 (46.7)*	25 (41.7)*	10 (16.7)*
Normal tissues with brain metastases	60	6 (10.0)#	8 (13.3)#	5 (8.3)#	3 (5.0)#	4 (6.7)#
Cancer tissues without brain metastases	120	40 (33.3)	45 (37.5)	32 (26.7)	33 (27.5)	47 (39.2)
Normal tissues without brain metastases	120	15 (12.5)	17 (14.2)	13 (10.8)	11 (9.2)	12 (10.0)

*brain metastases vs no brain metastases in cancer tissues, $p<0.05$; #brain metastases vs no brain metastases in adjacent normal tissue, $p>0.05$.

Table 3. RT-PCR results analysis

Group	*AEG-1*	*CXCR4*	*N-cadherin*	*a-SMA*	*E-cadherin*
Cancer tissues with brain metastases	0.4658±0.1235*	0.5526±0.1624*	0.6123±0.2132*	0.4869±0.2025*	0.1026±0.0652*
Normal tissues with brain metastases	0.1232±0.0564#	0.1625±0.0523#	0.2132±0.0629#	0.0659±0.0057#	0.0595±0.0062#
Cancer tissues without brain metastases	0.3262±0.1023	0.3524±0.1124	0.4215±0.1659	0.2659±0.1024	0.4568±0.1857
Normal tissues without brain metastases	0.1128±0.0629	0.1241±0.0427	0.2025±0.0852	0.0754±0.0052	0.0659±0.0033

* brain metastases vs no brain metastases in cancer tissues, $p<0.05$; #brain metastases vs no brain metastases in adjacent normal tissue, $p>0.05$

Table 4. Western blot results analysis

Group	*AEG-1*	*CXCR4*	*N-cadherin*	*α-SMA*	*E-cadherin*
Cancer tissues with brain metastases	0.45±0.13*	0.59±0.15*	0.63±0.21*	0.47±0.18*	0.12±0.05*
Normal tissues with brain metastases	0.11±0.05#	0.13±0.04#	0.20±0.07#	0.07±0.02#	0.05±0.01#
Cancer tissues without brain metastases	0.33±0.11	0.36±0.12	0.41±0.18	0.27±0.09	0.48±0.21
Normal tissues without brain metastases	0.12±0.04	0.13±0.05	0.23±0.13	0.08±0.02	0.07±0.02

* brain metastases vs no brain metastases in cancer tissues, $p<0.05$; #brain metastases vs no brain metastases in adjacent normal tissue, $p>0.05$

Discussion

This study showed that the expressions of AEG-1, CXCR4, N-cadherin and α-SMA mRNA were all significantly higher in patients with brain metastases than in those without brain metastases, while the expression of E-cadherin showed the opposite trend. There was no significant difference between the two groups in the adjacent normal tissues. This suggests that AEG-1 and CXCR4 are highly expressed in breast cancer tissues, activating and regulating the EMT pathway to participate in brain metastases.

Previous studies [11,12] suggest that the expression of AEG-1 is also closely related to TNM stage, pathological grade, therapeutic effect and survival. AEG-1 can be used as an early predictor of distant metastasis such as the brain and lung [13]. AEG-1 targeted inhibition can reduce the incidence of tumor recurrence and metastasis [14]. CXCL12 is the only natural chemokine known to bind and activate CXCR4. The CXCL12/CXCR4 signaling axis is involved in the development and progression of many tumors [15]. Using specific antibodies, selective peptides and siRNA to block the CXCL12/CXCR4 signal axis activation expression can significantly inhibit breast cancer metastasis to the lymph nodes and lung [16]. CXCR4 is involved in the metastasis of tumor cells to the target organ [17]. At the same time, CXCR4 is often highly expressed in common metastatic sites such as lymph nodes, lung, liver, brain and bone marrow, while its expression is low in the kidney [18,19]. E-cadherin is a marker of epithelial phenotype while N-cadherin, α-SMA and vimentin are markers of mesenchymal phenotype. The decreased expression of epithelial phenotypic markers and increased expression of mesenchymal phenotypic markers are consistent with the EMT pathway and tumor metastasis [20]. EMT plays a major role in tumor recurrence and metastasis in malignant tumors of epithelial origin [21].

This study used multiple detection methods to detect the expressions of AEG-1, CXCR4 and multiple EMT markers in cancer tissues and adjacent tissues from breast cancer patients with brain metastases. It was suggested that AEG-1, CXCR4 and EMT markers could offer an early prediction of tumor recurrence and metastasis and provide important targets for early intervention. Next, overexpression or silencing of target can be used to confirm the value of regulating the biological behavior of tumor and provide a theoretical basis for clinical application. Then we can investigate the specific regulation mechanism of AEG-1, CXCR4 and EMT. Finally, the sensitivity and specificity curves of AEG-1, CXCR4 and EMT marker molecules can be established in early detection and used to improve the value of clinical application.

Conflict of interests

The authors declare no confict of interests.

References

1. Lacey JV, Kreimer AR, Buys SS et al. Breast cancer epidemiology according to recognized breast cancer risk factors in the Prostate, Lung, Colorectal and Ovarian (PLCO) cancer screening trial cohort. BMC Cancer 2009;9:84.
2. Witzel I, Oliveira-Ferrer L, Pantel K, Müller V, Wikman H. Breast cancer brain metastases: biology and new clinical perspectives. Breast Cancer Res 2016;18:8.
3. Olofsson MH, Ueno T, Pan Y et al. Cytokeratin-18 is a useful serum biomarker for early determination of

response of breast carcinomas to chemotherapy. Clin Cancer Res 2007;13:3198-3206.

4. Xu X, Li S, Lin Y et al. MicroRNA-124-3p inhibits cell migration and invasion in bladder cancer cells by targeting ROCK1. J Transl Med 2013;11:276.
5. Sarkar D. AEG-1/MTDH/LYRIC in liver cancer. Adv Cancer Res 2013;120: 193-221.
6. Onoue T, Uchida D, Begum NM, Tomizuka Y, Yoshida H, Sato M. Epithelial-mesenchymal transition induced by the stromal cell-derived factor-1/CXCR4 system in oral squamous cell carcinoma cells. Int J Oncol 2006;29:1133-1138.
7. Brown DM, Ruoslahti E. Metadherin, a cell surface protein in breast tumors that mediates lung metastasis. Cancer Cell 2004;5:365-374.
8. Abozeid SM, Hathout RM, Abou-Aisha K. Silencing of the metastasis-linked gene, AEG-1, using siRNA-loaded cholamine surface-modified gelatin nanoparticles in the breast carcinoma cell line MCF-7. Colloids Surfaces B Biointerfaces 2016;145:607-616.
9. Burger JA, Kipps TJ. CXCR4. A key receptor in the crosstalk between tumor cells and their microenvironment. Blood 2006;107:1761-1767.
10. de Nigris F, Schiano C, Infante T, Napoli C. CXCR4 inhibitors: tumor vasculature and therapeutic challenges. Recent Pat Anticancer Drug Discov 2012;7: 251-264.
11. Erdem H, Gundogdu B, Ankarali H et al. Correlation of astrocyte elevated gene-1, basic-fibroblast growth factor, beta-catenin, Ki-67, tumor necrosis factor-alfa with prognostic parameters in ductal carcinomas and ductal intraepithelial neoplasms. Niger J Clin Pract 2015;18:638-643.
12. Zhang L, Yang Y, Wei XY et al. Reversing adriamycin resistance of human breast cancer cells by hyperthermia combined with Interferon alpha and Verapamil. J Exp Clin Cancer Res 2007;26:201-207.
13. West DC, Thompson WD, Sells PG, Burbridge MF. Angiogenesis assays using chick chorioallantoic membrane. Methods Mol Med 2001;46:107-129.
14. Lin F, Wang X, Wei J, Ren J, Zhang H. Serum anti-AEG-1 auto-antibody is a potential novel biomarker for malignant tumors. Oncol Lett 2012;4:319-323.
15. Chinni SR, Yamamoto H, Dong Z, Sabbota A, Bonfil RD, Cher ML. CXCL12/CXCR4 transactivates HER2 in lipid rafts of prostate cancer cells and promotes growth of metastatic deposits in bone. Mol Cancer Res 2008;6:446-457.
16. Platt D, Amara S, Mehta T et al. Violacein inhibits matrix metalloproteinase mediated CXCR4 expression: Potential anti-tumor effect in cancer invasion and metastasis. Biochem Biophys Res Commun 2014;455:107-112.
17. Smith MCP, Luker KE, Garbow JR et al. CXCR4 regulates growth of both primary and metastatic breast cancer. Cancer Res 2004;64:8604-8612.
18. Hinton CV, Avraham S, Avraham HK. Role of the CXCR4/CXCL12 signaling axis in breast cancer metastasis to the brain. Clin Exp Metastasis 2010;27:97-105.
19. Holland JD, Györffy B, Vogel R et al. Combined Wnt/β-Catenin, Met, and CXCL12/CXCR4 Signals Characterize Basal Breast Cancer and Predict Disease Outcome. Cell Rep 2013;5:1214-1227.
20. Park MH, Song B, Hong S, Kim S-H, Lee K. Biomimetic 3D clusters using human adipose derived mesenchymal stem cells and breast cancer cells: A study on migration and invasion of breast cancer cells. Mol Pharm 2016;13:2204-2213.
21. Blick T, Widodo E, Hugo H et al. Epithelial mesenchymal transition traits in human breast cancer cell lines. Clin Exp Metastasis 2008;25:629-642.