

Expression and Mechanism of Integrin Alpha 5 in Breast Cancer Bone Metastasis Model Rats

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Wang *et al.*: Mechanism of Integrin Alpha 5 in the Rat Model

To explore the role and possible mechanism of integrin alpha 5 in the rat model of bone metastasis of breast cancer. A lentiviral vector interfering with silencing the expression of integrin alpha 5 nucleotide sequence was constructed and transfected into breast cancer BT474 cell line as silent group, blank vector group and control group. The ability of cell growth was detected by cell counting kit-8 method, the ability of cell metastasis was measured by cell scratching method, the invasive ability of cells was determined by Transwell, the cell adhesion was measured by cell adhesion test, the expression of non-muscle myosin heavy chain II and p-S1943 protein was measured by Western blot, and the success rate of tumor bearing, the number and proportion of bone metastases were observed in rats with different carrier cells injected into the left ventricle. The cell survival rate, the number of cells reaching the basement membrane, the number of adherent cells, cell proliferation ability, and the expression of non-muscle myosin heavy chain II and p-S1943 in the silent group (group C) was reduced than that in the blank vector group (group B) and the control group (group A). The success rate of tumor-bearing and bone metastases was 100 % and 75.00 % in the rats injected with blank vector cells, but 71.432 % and 45.45 % in the rats injected with silenced integrin alpha 5 cells, respectively. Silent integrin alpha 5 can effectively inhibit the growth, metastasis and adhesion of breast cancer cells, and inhibit bone metastasis of breast cancer. The mechanism may be achieved by inhibiting the expression of non-muscle myosin heavy chain II and p-S1943 proteins.

Key words: Integrin alpha 5, bone metastasis, breast cancer, tumor, renal cell carcinoma

A malignant tumor known as Breast Cancer (BrCa) poses a major danger to the health of women, accounting for 30 % of the total incidence of female malignant tumors. The occurrence and development of BrCa is a complex process affected by heredity, environment, living habits and other factors, but the exact pathogenesis is not completely clear, and cancer cell metastasis is the main cause of death of BrCa^[1,2]. Cancer metastasis is a multi-step process, including adhesion and metastasis of tumor cells to Extracellular Matrix (ECM), extravasation to distant tissues and intravascular infiltration into the circulation system, resulting in micro metastasis^[3]. Tumor cells attach to the ECM and form protuberances through the FA on the anterior edge; traction causes the posterior edge's current cell-ECM contact to break down, allowing the cells to advance^[4].

In cell migration, Cell Adhesion Molecules (CAM) molecules play a crucial role. Integrin's are the major

group of CAM molecules^[5,6]. A total of 25 different heterodimer receptors are formed from the integrin family, which is composed of 18 glycoprotein subunits and 8 glycoprotein subunits. The proteins recognize and bind specific molecules of the ECM, transmit signals in both directions on the cell membrane, and sense environmental changes^[7]. It activates Focal Adhesion Kinase (FAK) when it binds to ECM ligands and regulates cell adhesion and movement. When FAK is activated, it attracts Src family kinases and auto phosphorylates its own tyrosine 397 residues, activating downstream signal effectors such Rac1^[7,8]. At the leading edge of mobile cells, actin polymerization mediated by Scar/Wiskott-Aldrich Syndrome Protein (WASP)

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and the development of flaky pseudopodia are regulated by Rac1^[9]. Activated integrin 5 forms a heterodimer with activated integrin 1. This heterodimer interacts with fibronectin to regulate cell adhesion^[10]. Human hepatocellular carcinoma cells are able to adhere and migrate *via* integrin 5. Up-regulation of integrin 5 indicates poor prognosis in BrCa^[11,12]. However, the role of integrin Alpha (α) 5 in BrCa cell proliferation and metastasis is not clear. The goal of this research is to explore the role of integrin α 5 in BrCa bone metastasis model rats and its possible mechanism, in order to bring a fresh concept for the mechanism of BrCa bone metastasis.

MATERIALS AND METHODS

General information:

Experimental animals: Totally 14 Sprague–Dawley (SD) nude mice and 8 mo old rats were selected. The license number of animal experimental facility is SCXK- (Beijing) 2012-0001.

Experimental cells: BrCa BT474 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10 % fetal bovine serum and 1 % penicillin-streptomycin and placed in 5 % Carbon dioxide (CO₂) incubator at 37°. The cell growth was observed and passaged once every 2 d to 4 d according to the cell growth and the color change of the medium. When the cells were fused to about 80 %, trypsin digestion was carried out, and inoculated in a 6-well plate, and the logarithmic growth phase was used.

Methods:

Vector construction and transfection: The lentiviral vector that interferes with the silencing of integrin α 5 nucleotide sequence was constructed and transfected into BrCa BT474 cell line as silent group, the BrCa BT474 cell line transfected with blank vector as blank vector group, and the untreated BrCa BT474 cell line as control group.

Model building: The left ventricular injection model of BrCa cells was designed. After anesthesia, the solution prepared by BrCa BT474 cells was injected into the second and third ribs of mice. After 10 min, the cells were observed by *in vivo* imaging, and the cells circulated to the whole body blood system, indicating that the left ventricle injection was successful. Bone metastasis was

examined by animal *in vivo* imaging and X-ray.

Observation index:

Cell proliferation: The ability of cell proliferation was detected by Cell Counting Kit-8 (CCK-8) method. The vigorous cells in logarithmic phase were selected and fully mixed into cell suspension for culture. After 24 h of culture, CCK-8 reagent 10 μ l was added into each well, and then cultured for 24 h. The Optical Density (OD) value of each well was detected and the cell density of each hole was evaluated.

Cell migration: The ability of cell migration was determined by cell scratch method. The cells of each group were cultured in serum-free medium for 1 d. After trypsin digestion, the Roswell Park Memorial Institute (RPMI)-1640 cell culture medium was re-suspended and inoculated to a 6-well plate according to 5×10^4 /well. When the cells grew well, the residual serum medium was washed off by Phosphate Buffer Solution (PBS), and a straight line was drawn at the bottom of the orifice plate with the gun head, and the image was recorded under the microscope and incubated for 24 h. The image is collected again under the microscope, the actual migration distance is measured with the measuring tool, and the mobility is calculated.

Transwell chamber invasion experiment: 50 μ l diluted Matrigel glue was added to each transwell room, and slowly added from the edge, and then solidified in the incubator at 37° for 0.5 h. The cells were cultured for 48 h after inoculation. Remove the upper chamber, fix the cells with 4 % methanol and rinse with PBS for 15 min. 2 % crystal violet staining for 15 min PBS cleaning 3 times. Observed and photographed under the microscope, counted the number of cells on the lower surface of the filter membrane, and expressed the invasive ability of the corresponding cells by the number of transmembrane cells.

Cell adhesion experiment: After being cultured for 24 h, each group of cells was digested with trypsin without Ethylenediaminetetraacetic Acid (EDTA) and centrifuged, then washed with PBS twice to make 6×10^4 /ml single cell suspension, 96-well flat-bottomed cell culture plate with 100 μ l single cell suspension, away from light, incubated at 37° for 1 h, sucked out the culture medium,

washed 3 times, removed non-adherent cells, and then made into DMEM solution, incubated for 1 h. Finally, enzyme labeling instrument was used to detect the absorbance of cells in each group.

Western blot: The expression of Non-Myosin Heavy Chain (NMHC) II and p-S1943 protein was measured by Western blot. After 24 h of treatment, the cells in each group were washed with PBS solution, and the total protein was extracted.

Experimental analysis: Experimental analysis of tumor-bearing animals with bone metastasis of BrCa. Statistics on the success rate of tumor-bearing, the number and proportion of bone metastases in rats with left ventricular injection of different carrier cells into the tumor *in vivo*.

Data processing:

The research data are analyzed by Statistical Package for the Social Sciences (SPSS) 20.0 software package, all measurement data that follow the normal distribution are compared using the ($\bar{x}\pm s$) method, expression of the comparison between groups by single factor analysis of variance, the pairwise comparison uses Student–

Newman–Keuls (SNK)-q test; a statistical test was used to compare the counts between groups. The results were statistically significant $p<0.05$.

RESULTS AND DISCUSSION

The cell survival rate in the silent group (group C) was reduced than the blank vector group (group B) and the control group (group A) (Table 1). Compared to group B and group A, group C had fewer cells that reached the basement membrane (Table 2). The number of adherent cells in the group C was reduced than the group B and the group A (Table 3). The cell proliferation ability of the group C was reduced than that of the group B and the group A (Table 4). Compared with the group B and the group A, the NMHC II and p-S1943 in the group C was lower than the group A (Table 5).

The success rate of tumor-bearing and bone metastases was 100 % and 75.00 % in the rats injected with blank vector cells, but 71.432 % and 45.45 % in the rats injected with silenced integrin α -5 cells, respectively. The success rate and the proportion of bone metastases in the silent integrin α -5 group were reduced than the group B (Table 6).

TABLE 1: CELL MIGRATION

| Group | n | Cell migration rate |
|-------|---|--------------------------|
| A | 6 | 43.68±10.67 |
| B | 6 | 41.52±12.54 |
| C | 6 | 12.57±5.85 ^{ab} |
| F | | 35.244 |
| P | | <0.001 |

Note: ^a $p<0.05$ compared to the blank control group, and ^b $p<0.05$ compared to the hypoxia group

TABLE 2: COMPARISON OF INVASIVENESS OF CELLS IN DIFFERENT GROUPS

| Group | n | Invasiveness rate ($\times 10^4$) |
|-------|---|-------------------------------------|
| A | 6 | 4.26±1.21 |
| B | 6 | 4.08±1.16 |
| C | 6 | 2.27±1.24 ^{ab} |
| F | | 16.372 |
| P | | <0.001 |

Note: ^a $p<0.05$ compared to the blank control group, and ^b $p<0.05$ compared to the hypoxia group

TABLE 3: COMPARISON OF CELL ADHESION AMONG DIFFERENT GROUPS

| Group | n | Cell adhesion rate |
|--------------|---|-------------------------|
| Control | 6 | 0.43±0.04 |
| Blank vector | 6 | 0.46±0.05 |
| Silent | 6 | 0.37±0.02 ^{ab} |
| F | | 6.587 |
| P | | <0.001 |

Note: ^a $p<0.05$ compared to the blank control group, and ^b $p<0.05$ compared to the hypoxia group

TABLE 4: COMPARISON OF CELL PROLIFERATION AMONG DIFFERENT GROUPS

| Group | n | Cell proliferation rate |
|-------|---|-------------------------|
| A | 6 | 1.75±0.18 |
| B | 6 | 1.68±0.19 |
| C | 6 | 1.38±0.21 ^{ab} |
| F | | 5.983 |
| p | | <0.001 |

Note: ^ap<0.05 compared to the blank control group, and ^bp<0.05 compared to the hypoxia group

TABLE 5: COMPARISON OF PROTEIN EXPRESSION OF PI3K/AKT PATHWAY IN CARDIOMYOCYTES OF RATS IN DIFFERENT GROUPS ($\bar{x}\pm s$)

| Group | n | NMHCII | p-S1943 |
|--------------|---|-------------------------|-------------------------|
| Control | 6 | 0.58±0.15 | 1.03±0.08 |
| Blank vector | 6 | 0.61±0.13 | 0.97±0.12 |
| Silent | 6 | 0.17±0.08 ^{ab} | 0.53±0.09 ^{ab} |
| F | | 6.823 | 5.954 |
| p | | <0.01 | <0.01 |

Note: ^ap<0.05 compared to the blank control group, and ^bp<0.05 compared to the hypoxia group

TABLE 6: EXPERIMENTAL ANALYSIS OF INTEGRIN α -5 REGULATING BONE METASTASIS OF BRCA IN ANIMAL

| Group | Tumor formation/ tumor-bearing (only) | Tumor-bearing Success rate | Metastatic focus Number | Number of bone metastases | Proportion of bone metastases |
|----------------------------------|--|-------------------------------|----------------------------|------------------------------|----------------------------------|
| B | 7/7 | 100 % | 24 | 18 | 75.00 % |
| Silenced integrin α -5 | 5/7 | 71.43 % | 11 | 5 | 45.45 % |

Integrin, especially subunit integrin α -5, is an effective trigger for the progression and metastasis of many kinds of solid tumors. In glioma cells, integrin α -5 is related to the enhanced ability of cell migration^[13]. In addition, Gong *et al.*^[14] found that integrin α -5 induces Epithelial-Mesenchymal Transition (EMT) and promotes cancer cell metastasis in Head and Neck Squamous Cell Carcinomas (HNSCCs). In Renal Cell Carcinoma (RCC), Sawada *et al.*^[15] confirmed that integrin α -5 plays a role in the progression of RCC. As well as its expression being twice as high in RCC as in benign renal tissue, it was also higher in high-grade tumors than in low-grade tumors. Integrin α -5 contributes to the growth and progression of clear cell RCC (ccRCC) tumors. High integrin α -5 is connected to poor overall survival and lymph node metastasis^[16]. The correlation between the expression of integrin α -5 and prognosis was also confirmed in ovarian cancer^[17]. Lung cancer and high-grade gliomas showed similar results, suggesting that integrin α -5 may have carcinogenic characteristics^[18].

The most crucial stage in the development of a tumor is cancer cell metastasis, which ultimately

results in death. As tumor cells metastasize, they enter the systemic circulation, extravasate to distant niches, and proliferate in secondary organs. Cell metastasis and adherence to ECM are crucial phases in the metastatic process. As a consequence, metastasis may be influenced by membrane-bound receptors, such as integrin α -5, that identify ECM components. In earlier investigations^[19], proposed the relationship between integrin α -5 and bone specific metastasis of RCC. In primary ccRCC cells taken from patients who developed bone metastases within 5 y of surgery, integrin α -5 expression was greater than in those who did not develop bone metastasis. In this study, the effects of BrCa integrin α -5 on cell adhesion and metastasis were analyzed. The silencing of integrin α -5 decreased the cell adhesion of BrCa cells and decreased the rate of bone metastasis in animal experiments. The inhibition of integrin α -5 in colon cancer cells led to a decrease in cell adhesion of Caki-1 and CCF-RC1 cells and a decrease in chemotactic migration to fibronectin. It is recommended that integrin α 5 plays critical role in extravasation and invasion of BrCa cells during metastasis.

In RCC, CCF-RC1 cell line, integrin can activate

Extracellular-Regulated Kinase (ERK) through FAK and Shc, thus inducing cell adhesion and migration^[20]. Integrin β 1-FAK axis regulates cell migration in CCF-RC1 cells^[21]. It can be seen that the transfer of ccRCC is mediated by integrin α -5 and ERK signal pathway is activated by Shc and FAK. However, the mechanism of action in BrCa cells is not clear. NMHC is an important component of non-myosin II. It can bind to actin and regulate cell movement directly through auto phosphorylation. Human NMHC II includes three subtypes; NMHCIIA, NMHCIIB and NMHCIIC. NMHC II is encoded by Myosin-9 (MYH9) gene. Previous studies have confirmed that NMHC II serves a crucial function in pathophysiological processes such as immune response, cell migration, adhesion and tumor metastasis^[22]. In papillary thyroid carcinoma, NMHC II inhibits the common promoter of Forkhead Box E1 (FOXE1) and Papillary Thyroid Cancer Susceptibility Candidate 2 (PTCSC2) by binding to long non-coding RNA (lncRNA)-PTCSC2, thus inhibiting p53 pathway and promoting cancer progression. Coincidentally, the overexpression of NMHC II is related to the metastasis of prostate cancer. Moreover, NMHC II may modulate the metastasis of prostate cancer by regulating the migration, invasion and proliferation of prostate cancer cells. However, it is not clear whether NMHC II plays a regulatory role in BrCa. In this study, it was found that the expression of NMHC II and p-S1943 in silent group was reduced than the group B and group A. According to the analysis of integrin structure and tumor cell invasion characteristics, integrin may participate in the regulation of cell invasion and migration through its special transmembrane structure, realizing the dual regulation of mechanical and chemical signal pathways, i.e. the extracellular region of integrin receives extracellular chemical signals, converts them into mechanical signals, and then transmits them to NMHC cytoskeleton proteins through the intracellular domain, triggering the activation of NMHC, leading to NMHC assembly. This leads to a series of changes in the biological behavior of cells.

To sum up, silent integrin α -5 can effectively inhibit the growth, metastasis and adhesion of BrCa cells, and inhibit bone metastasis of BrCa. The mechanism may be achieved by inhibiting the expression of NMHC II and p-S1943 proteins.

Author's contributions:

Gang Wang and Bangliang Xia have contributed equally to this work.

Conflict of interests:

The authors declared no conflict of interests.

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