# The relationship between the expressions of tumor associated fibroblasts Cav-1 and MCT4 and the prognosis of papillary carcinoma of breast

**Yuanyuan Zhai, Lijun Chai and Jianzhong Chen***
Department of Galactophore, The Second Affiliated Hospital of Zhengzhou University, Zhengzhou, China

**Abstract**: The aim of this study was to evaluate the expression levels of Cav-1 and MCT4 in the tumor stroma CAFs. Immunohistochemical method was used to detect the expression of Cav-1 and MCT4 in 86 cases of IMPC, with 105 cases of non-specific invasive ductal carcinoma (IDC-NOS) as control. Their differential expression in different histological types were compared and relationship between the expression of Cav-1 and IMPC in the MCT4 and the main pathological features such as age, tumor size and histological grades were analyzed. The study confirmed that the loss expression of Cav-1 on CAFs and the up-regulation of MCT4 may be the possible mechanisms of CAFs in tumorigenesis, which may be one of the molecular mechanisms leading to the poor prognosis of IMPC. The expression of CAFs related proteins in tumor microenvironment suggested that CAFs could be used as the target of tumor therapy and provide new evidence for the treatment of breast cancer.

**Keywords**: Breast cancer; CAFs; Cav-1; MCT4.

## INTRODUCTION

Breast cancer is a kind of highly heterogeneous malignant tumor, which is characterized by different histological types with different biological characteristics and clinical prognosis. Therefore, the full understanding of various histological types of breast cancer, and the study of the occurrence and development of different types of breast cancer and the mechanism of invasion and metastasis, would be of great significance to understand the biological characteristics of breast cancer and its clinical diagnosis, treatment and prognosis. So it is possible to improve the overall level of breast cancer treatment to some extent. Since 1999, our group has been working on the relationship between morphology and biological behavior of Invasive micro papillary carcinoma (IMPC). Our preliminary study (Guo *et al*., 2006; Fu *et al*., 2004a; Fu *et al.*, 2004b) found that the IMPC tumor cell clusters existed in primary foci, invasion of lymphatic vessels and metastasis in a same form of small nipple or micro tube structure. IMPC can occur in the breast, lung, ovary, liver and other organs (Cha *et al*., 2014). The special growth patterns and high metastatic malignancies of IMPC and other poor prognosis of malignant biological characteristics provides a good model for us to study the mechanism of tumor invasion and metastasis. Our previous study also found that the stroma contains a large number of myofibroblasts and newborn capillaries (Fu *et al*., 2004). Well, it is not clear that how much of these large number of mesenchymal cells in the tumor microenvironment will affect the occurrence, development and biological behavior of IMPC. It's also not clear that the expression of Cav-1 and IMPC in carcinoma-associated fibroblasts (CAFs) and its correlation with clinicopathological parameters and its relationship with histological types of breast cancer. In this study, by detecting the differential expression of Cav-1 and MCT4 in IMPC and invasive ductal carcinoma, not otherwise specified (IDC-NOS), the relationship between the expression of these two proteins and the prognosis of IMPC was analyzed to explore the molecular basis of IMPC growth, invasion and metastasis from the point of view of the tumor microenvironment, providing new and valuable auxiliary indexes for the prognosis of breast cancer.

**Corresponding author:* e-mail: zmabc2015@sina.com

## MATERIALS AND METHODS

### *Objection and method*

*The agents*

Mouse anti human Cav-1 monoclonal IgG antibody (Abeam, Englishi, 1:200); Rabbit anti human MCT4 polyclonal IgG antibody (Santa cruz Biotechnology, America, 1:100); Mouse anti human a-SMA monoclonal IgG antibody (mouse IgQ Zymed, USA, 1:50); Rabbit anti human Estrogen receptor monoclonal IgG antibody (clone SP1, Zymed, America); Rabbit anti human progesterone receptor monoclonal IgG antibody (clone SP2, ZYmed, America); Mouse anti human HER-2/neu monoclonal antibody (DAKO Hercep Test TM, Denmark); Mouse anti human EMA (epithelial membrane antigen) monoclonal IgG antibody (DAKO, Denmark, 1:100); PBS phosphate buffer salt (0.01 M pH 7.27-7.4 XZLI-9062, Beijing Zhongshan Jinqiao Biological Technology Co., Ltd); Citrate repair solution (PH 6.0, 0.01M) (ZLI-9065, Beijing Zhongshan Jinqiao Biological Technology Co., Ltd); EDTA repair solution (1 mM PH 9.0) (ZLI-9067, Beijing Zhongshan Jinqiao Biological

Technology Co., Ltd); Blocked with normal goat serum working solution (SP-9000, Beijing Zhongshan Jinqiao Biological Technology Co., Ltd); Antibody dilution (ZLI-9030, Beijing Zhongshan Jinqiao Biological Technology Co., Ltd); Biotin labeled second antibody (SP-9000, Beijing Zhongshan Jinqiao Biological Technology Co., Ltd); Chain avidin of working fluid labeled with horseradish peroxidase (SP-9000, Beijing Zhongshan Jinqiao Biological Technology Co., Ltd); Endogenous peroxidase blocker (SP-9000, Beijing Zhongshan Jinqiao Biological Technology Co., Ltd); DAB HRP-OPD (ZLI-9032, Beijing Zhongshan Jinqiao Biological Technology Co., Ltd); Netrual balsam (ZLI-9055, Beijing Zhongshan Jinqiao Biological Technology Co., Ltd); APES (ZLI-9001, Beijing Zhongshan Jinqiao Biological Technology Co., Ltd); Xylene (Tianjin Fangde Technology Co., Ltd); Absolute ethyl alcohol (Tianjin Fangde Technology Co, Ltd).

***Main instrument***

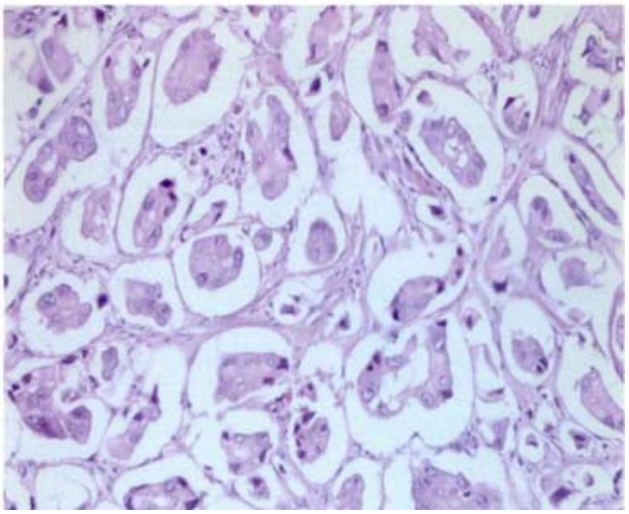
Paraffin section machine (RM2235, LEICA, German); Electro-heating standing-temperature cultivator (HHB1M20, Tianjin experimental instrument factory); Stainless steel pressure cooker; Medical centrifuge (Shanghai Medical Instrument Co., Ltd); Optical microscope (BX60, Olympus, Japan); TK-C1381 image acquisition system (JVC, Japan).

***Histopathological diagnosis analysis***

Diagnosis of IMPC: IMPC was diagnosed according to the morphological criteria of WHO breast cancer classification in 2003 and 2012 (Reis-Filho and Ellis, 2012; Tavassaoeli and Davilee, 2003): IMPC was morphologically characterized by a small nipple or a polar inverted tubular structure consisting of a number of tumor cells without fiber bundles (fig. 1). Our previous studies have confirmed that the proportion of IMPC in tumor and lymph node metastasis had no significant correlation (Liu *et al*., 2009). Therefore, IMPC could be diagnosed as long as the cancer nest contained a typical IMPC components. IMPC components in all cases were strongly expressed in the connection between cancer cell clusters through epithelial membrane antigen, the positive expression site in the micro-papillary or the outside of false glandular, that was, the interstitial side of cancer cell clusters and the E-cadherin, but not expressed in the outer side of the cancer cell mass, that was, interstitial connection surface (figs. 2, 3).

Tumor size in IMPC and IDC-NOS cases: The maximum diameter of IMPC and IDC-NOS was recorded. Tumor size staging: T1 staging: the maximum diameter ≤2cm; T2 staging: the maximum diameter >2cm, <5cm;T3 staging: the maximum diameter >5cm.

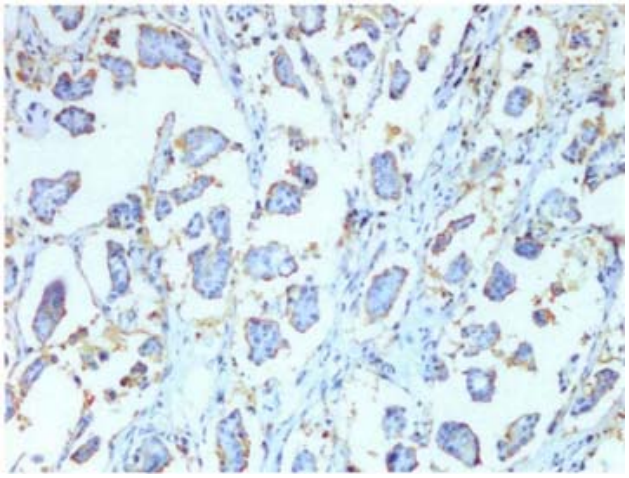
Histopathological grading of IMPC and IDC-NOS cases: I: The size, shape and chromatin of tumor cells were basically the same or slightly abnormal, and there were no or even 1 mitotic figs. 10 HPF; II: The size and shape of the tumor cell nucleus and the chromatin were moderately abnormal, and the 2-3 mitotic figs. could be seen/10HPF; III: The size and shape of the tumor cell nucleus, and the chromatin were highly heterogeneous, showing >3 mitotic figs. 10HPF.



Note: The tumor cells were arranged into a small papillary or polar inverted tubular like structure without a fibrous bundle

**Fig. 1**: Morphological expression of IMPC (HE staining)

The degree of lymph node metastasis of IMPC and IDC-NOS was divided into 4 grades according to the number of metastasis (NO: 0, N1: 1-3, N2: 4-9, N3: >9).



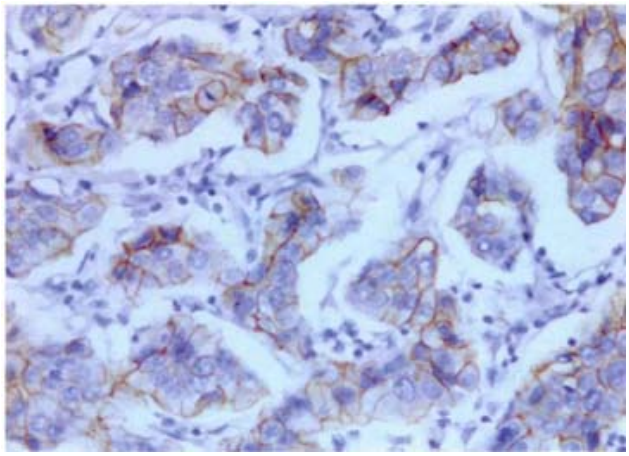
Note: The positive expression was located in the outer part of the papillary or pseudo duct, which was the interstitial side of cancer cell mass.

**Fig. 2**: EMA immunohistochemical staining of IMPC

***The research objects***

86 cases of IMPC admitted to the Tumor Hospital Affiliated to Tianjin Medical Univercity from January 2003 to August 2005 were selected breast cancer specimens. The patients were all female and the median age was 52 years (28-82). At the same time, we randomly

selected 105 cases of IDC-NOS in the same period as the control group. All the specimens were fixed by 10% neutral formalin and embedded in paraffin. All patients received no asjuvant therapy before surgery.



Note: The positive expression sites were strongly expressed on the surface of cancer cells.

**Fig. 3**: ECD immuohistochemical staining of IMPC

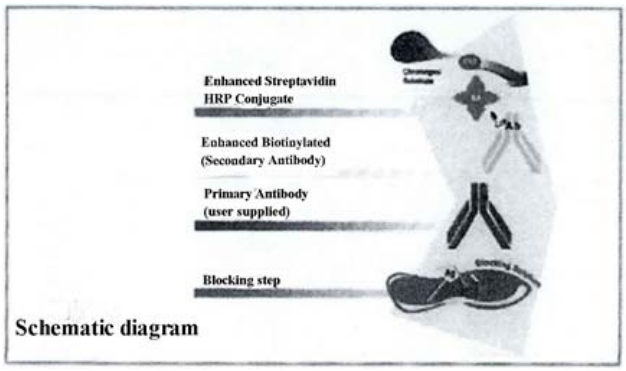



**Fig. 4**: Schematic diagram of SP method

The selected IMPC patients were followed up for 1-115 months, and the median follow-up time was 64 months. The survival status of patients included death and recurrence, which were mainly obtained by telephone, letters, visites and access to medical records. The recurrence of the tumor was mainly local recurrence (ipsilateral locking, chest wall recurrence and internal mammary lymph node metastasis), new occurrence of contralateral and the distant metastasis (contralateral locking, cervical lymph nodes, liver, bone, lung, brain and other distant organ metastasis). The data of the patients who died of breast cancer were complete data, and the data of survival or death were defined as censored data. The progression-free survival (PFS) of patients was defined as the time from the date of surgery to the time of the first tumor recurrence or the last follow-up time. The overall survival (OS) was defined as the time from surgery to the time of death or last follow-up. During the follow-up period, 34 patients had tumor recurrence, and only 8 died of breast cancer.

***Immunohistochemical staining***

The expression of Cav-1 and MCT4 in IMPC and IDC-NOS in breast cancer were detected by Labeled Streptavidin Biotin (LSAB/SP). PBS buffer was used as a negative control, and known positive. The staining results were dyed with DAB.

***The basic principle***

The immunohistochemical method adopted in this study is a highly sensitive affinity histochemical technique, namely, the enzyme labeled streptavidin-biotin (LSAB) technique. Biotin, also known asvitamin H, was a small molecule vitamin and its molecular weight was 244; the Stretavidin (SA) was a protein isolated from Streptomyces, with a molecular weight of 60 KD. There were 4 biotin binding sites in one molecule, which structure was similar to that of antibiotin in the ovi albumin, but it could exclude the non-specific binding of the latter to the endogenous substance in the tissue. Thus, the background color was shallow and could obtain higher signal-to noise ratio (fig. 4).

1) Slide processing: The slides were soaked in soapy water overnight and washed with running water, then soaked with 95% alcohol soaked overnight and dried. The cleaned slides were placed in the newly prepared APES acetone working solution (1:50) for 20-60 seconds, removed for a short pause, and then placed in pure acetone solution or distilled water to rinse the unbound APES, then dried and saved in box. 2) The paraffin-embedded tissue sections were sliced to a thickness of about 4 μm, baked on a baking machine for 2 h, and then placed in a 60□ incubator overnight to reduce release. 3) Paraffin sections were dewaxed to water by conventional procedures. 4) Washed with distilled water and soaked into PBS for 5 min. 5). The paraffin sections were processed with antigen thermal remediation (citrate acid, PH 6.0, high pressure repair for 2 min) and cooled to room temperature. 6) Washed 3 times with PBS for 5 min each time. 7) 3% of the peroxygen peroxide was incubated at room temperature for 10 minutes to eliminate endogenous peroxidase activity. 8) Washed 3 times with PBS for 5 min each time. 9) Closed with the normal serum of rabbit/rat and incubated for 10 min at room temperature, and poured without washing. 10) Primary antibody (Cav-1 monoclonal antibody 1:200 dilution, MCT4 polyclonal antibody 1:100 dilution) was dropped at 4$^{o}$C overnight. PBS was used instead of primary antibody as a negative control. 11) Washed with PBS for 3 times and each for 5 min. 12) Biotinylated secondary antibody solution was added and incubated at 37$^{o}$C for 20 min, 13) Washed with PBS for 3 times, and each for 5 min. 14) Horseradish enzyme labeled streptavidin three anti-working solution was added and incubated for 20 min at 37$^{o}$C. 15) Washed with PBS for 3 times, each for 5 min. 16) Dyed with new DAB reagents for 3-10 min at room temperature, observed under microscope, and washed with water to terminate the reaction. 17) Counterstained

**Table 1**: The dilution concentration of primary antibody and antigen repair conditions

| Antibody | Producer | Dilution | Antigen repair conditions |
|---|---|---|---|
| Cav-1 | Abcam | 1:200 | PH6.0 citrate high-pressure repair |
| MCT4 | Abcam | 1:100 | PH6.0 citrate high-pressure repair |
| EMA | DAKO | 1:100 | PH6.0 citrate high-pressure repair |
| α-SMA | Zymed | 1:150 | PH6.0 citrate high-pressure repair |
| ER | Zymed | 1:100 | PH6.0 citrate high-pressure repair |
| PR | Zmyed | 1:150 | PH6.0 citrate high-pressure repair |
| HER-2 | DAKO | 1:400 | PH6.0 citrate high-pressure repair |

**Table 2**: Comparison of clinical pathological features of IMPC and IDC-NOS in the breast

| Clinical pathological features | IMPC | IDC-NOS | Z value | *P |
|---|---|---|---|---|
| Age | | | | |
| ≤52 | 44(51.2) | 55(52.4) | -0.167 | 0.867 |
| >52 | 41(48.8) | 50(47.6) | | |
| Size tumor (T-stage) | | | | |
| T1 | 14(16.7) | 26(24.8) | -1.709 | 0.073 |
| T2 | 50(59.5) | 63(60.0) | | |
| T3-T4 | 20(23.8) | 16(15.2) | | |
| Histological grade | | | | |
| I | 7(8.1) | 6(5.7) | -1.104 | 0.270 |
| II | 54(62.8) | 79(75.2) | | |
| III | 25(29.1) | 20(19.1) | | |
| Lymph node staging | | | | |
| N0 | 12(14.1) | 38(36.6) | -4.515 | <0.001 |
| N1 | 17(20.0) | 31(29.8) | | |
| N2 | 20(23.5) | 15(14.4) | | |
| N3 | 36(42.438) | 20(19.2) | | |
| ER stage | | | | |
| Negative | 21(24.7) | 35(33.7) | -1.337 | 0.918 |
| Positive | 64(75.3) | 69(66.3) | | |
| PR stage | | | | |
| Negative | 29(34.1) | 41(39.4) | -0.749 | 0.804 |
| Positive | 56(65.9) | 63(60.6) | | |
| HER-2 stage | | | | |
| Negative | 69(82.1) | 77(76.2) | -0.978 | 0.328 |
| Positive | 15(17.9) | 24(23.8) | | |

*Mann-Whitney U test.

with hematoxylin, dehydrated and sealed with neutral gum.

2) Cav-1 protein: The expression of Cav-1 existed in tumor cells and tumor stroma. Cav-1 was mainly located in the cell membrane and cytoplasm, showing brownish yellow granules. The expression of Cav-1 in invasive carcinoma stroma is mainly focused on CAFs, and the CAFs was identified by α-SMA staining on the basis of its characteristic spindle structure. Cav-1 is mainly located in cytoplasm in CAFs. The expression of Cav-1 in the tumor epithelial cells and tumor matrix CAFs were analyzed respectively. The grade was determined according to the percentage of positive cells, that was, positive cells ≤10% was negative, positive cells between 10-30% was (+) and more than 30% (++).

2) MCT4 protein: There were expression of MCT4 in tumor cells and tumor matrix. MCT4 in tumor cells was mainly located in cell membrane and cytoplasm, showing brownish yellow granular. The expression and evaluation of MCT4 in invasive carcinoma stroma was mainly focused on CAFs, and the CAFs was identified by α - SMA staining on the basis of its characteristic spindle structure. MCT4 was mainly located in cytoplasm in the CAFs. The expression of MCT4 in tumor epithelial cells and tumor stroma CAFs was interpreted. The expression of MCT4 in tumor cells was determined by taking into account the staining intensity and proportion of positive cells. The positive intensity was divided into 0 point for colorless, 1 point for light coloring, 2 points for medium coloring, 3 points for strong coloring. The percentage of positive cells was divided into 0 point for 0% cells

**Table 3**: The comparison of expression of Cav-1 in tumor stroma and epithelium in IMPC cases

| | Stroma | Epithelium | Z | *P |
|---|---|---|---|---|
| Cav-1stage | | | | |
| - | 49(57) | 74(86) | -4.211 | <0.001 |
| +/++ | 37(43) | 12(14) | | |

**Table 4**: The comparison of expression of Cav-1 in tumor stroma and epithelium in IDC-NOS cases

| | Stroma | Epithelium | Z | *P |
|---|---|---|---|---|
| Cav-1stage | | | | |
| - | 38(36.2) | 91(86.7) | -7.496 | <0.001 |
| +/++ | 67(63.8) | 14(13.3) | | |

**Table 5**: The comparison of the expression of Cav-1 and MCT4 in IMPC and IDC-NOS cases

| | IMPC | IDC-NOS | Z value | *P |
|---|---|---|---|---|
| Tumor epithelium Cav-1 | | | | |
| - | 74(86) | 91(86.7) | -0.124 | 0.901 |
| +/++ | 12(14) | 14(13.3) | | |
| Stroma CAFsCav-1 | | | | |
| - | 49(57) | 38(36.2) | -2.862 | 0.004 |
| +/++ | 37(43) | 67(63.8) | | |
| Tumor epithelium MCT4 | | | | |
| T(-/+) | 38(44.2) | 55(52.4) | -1.124 | 0.261 |
| T(++) | 48(55.8) | 50(47.6) | | |
| Stroma CAFsMCT4 | | | | |
| S(-/+) | 27(31.4) | 54(51.4) | -2.708 | 0.005 |
| S(+/++) | 59(68.6) | 51(48.6) | | |

*Mann-Whitney U test.

**Table 6**: Relationship between epithelial and stromal expression of MCT4 in breast cancer patients with IMPC

| | The expression of epithelium | | | |
|---|---|---|---|---|
| | -/+ | ++ | $r_s$ | *P |
| The expression of stroma | | | | |
| -/+ | 16(59.4) | 11(22.9) | 0.205 | 0.058 |
| ++ | 22(57.9) | 37(7.1) | | |

*Spearman's Rank-Correlation test.

staining, 1 point for 5% cells staining, 2 points for 5-50% cells staining and 3 points for >50% cells staining. Positive intensity and proportion of positive cells were added to the final score: 0-2 was negative, 3-4 (+) and 5-6 (++). The expression of MCT4 in tumor matrix CAFs was determined by the percentage of positive cells, that was, the percentage of cells positive less than 10% was negative, between 10%-30% (+) and more than 30% (++).

3) The determination of ER and PR staining results (Nam *et al*., 2013): The expression of PR and ER was mainly located in nucleus with brownish yellow granular. The number of positive cells in the slice is greater than 1% of the number of tumor cells is defined as positive, less than 1% negative.

4) The determination of HER-2 staining results (Martinez-Outschoorn *et al*., 2010): The staining of positive cells was mainly localized in the cell membrane and the infiltrating tumor cells without cell membrane staining for (-); Any proportion of infiltrating tumor cells showed weak and incomplete cell membrane coloration or <10% of the tumor cells showed weak but incomplete cell membrane staining for (+); ≥ 10% infiltrating tumor cells showed weak or inconsistent but complete membrane staining, or ≤ 30% infiltrating tumor cells showed strong and complete membrane staining for (++); > 30 infiltrating tumor cells showed strong and complete membrane staining for (+++).

According to the actual situation of clinical application, this experiment would defined 0 and (+) as the HER-2 negative; (+++) was defined as HER-2 positive while the cases of (++) group were analyzed by fluorescence in situ hybridization (FISH) detection.

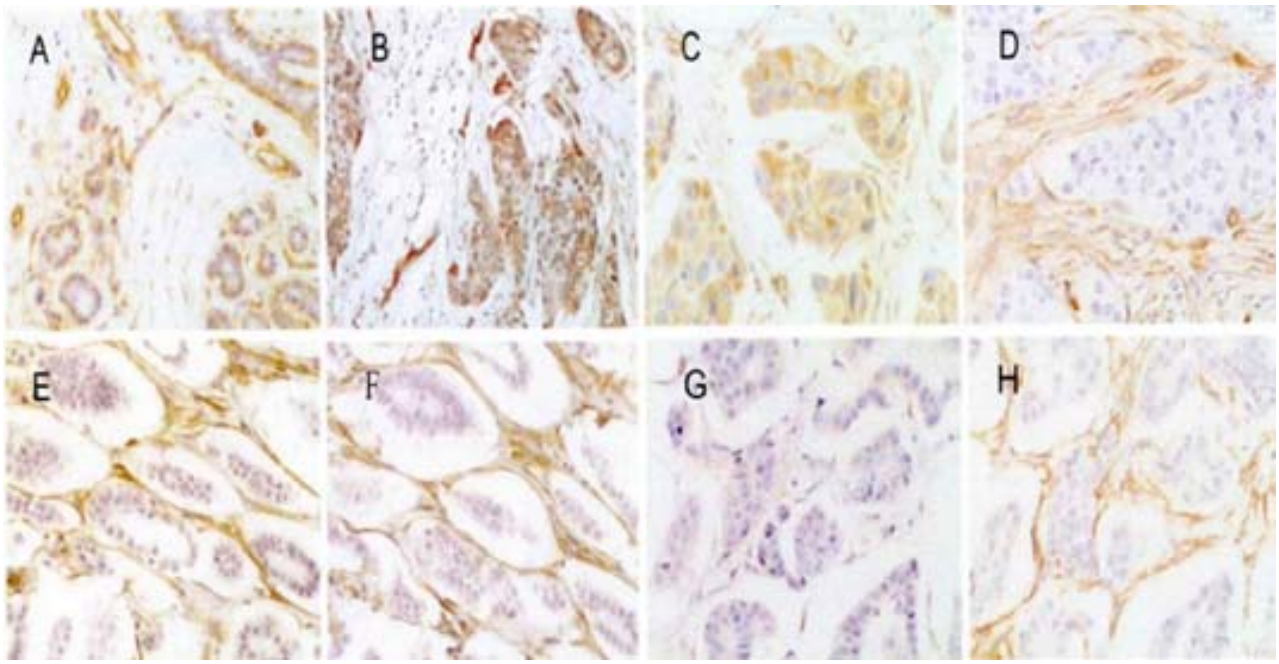



**Fig. 5**: Immunohistochemical staining of Cav-1 and α-SMA. Cav-1 staining was observed in lobular epithelial cells of normal breast tissue, myoepithelial cells around the lobular acini and vascular endothelial cells (A); the positive expression of Cav-1 in tumor epithelium of IDC-NOS (B); the positive expression of Cav-1 in tumor epithelium of IMPC (C); the positive expression of Cav-1 in tumor stroma of IDC-NOS (D); the positive of Cav-1 in tumor stroma CAFs of IMPC (E); through the serial section of α-SMA positive staining (F); the negative expression of Cav-1 in tumor stroma CAFs (G); the determined of CAFs through the serial section of α-SMA positive staining (H).

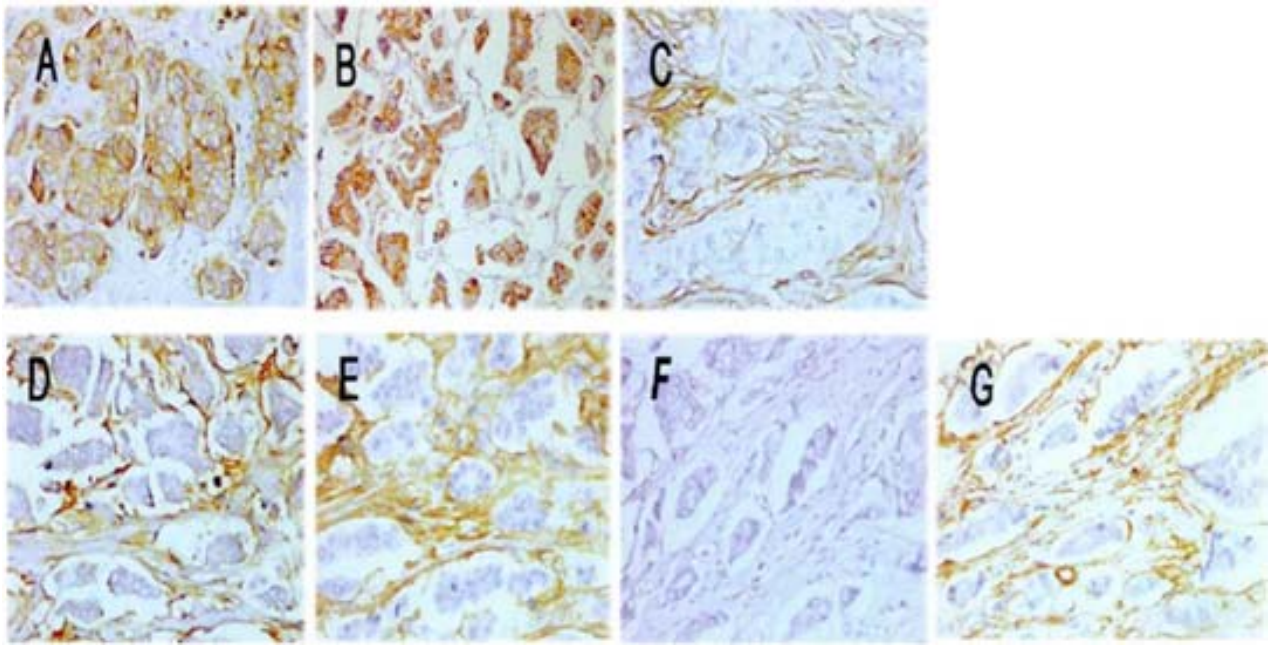



**Fig. 6**: Immunohistochemical staining of MCT4 and α-SMA. The negative expression of MCT4 in tumor epithelium cells (A); in IMPC, the positive expression of MCT4 in tumor epithelium (B); in IDC-NOS, the positive expression of MCT4 in tumor stroma (C); in IMPC, the positive expression of MCT4 of tumor stroma CAFs (D); through the serial section of α-SMA positive staining(E); in IMPC, the negative expression of MCT4 in tumor stroma CAFs (F); the determined of CAFs through the serial section of α-SMA positive staning (G).

**Table 7**: Relationship between epithelial and stromal expression of MCT4 in breast IDC-NOS cases

| | The expression of epithelium | | | |
|---|---|---|---|---|
| | -/+ | ++ | $r_s$ | *P |
| The expression of stroma | | | | |
| -/+ | 25 (45.5) | 29 (58) | -0.125 | 0.203 |
| ++ | 30 (54.5) | 21 (42) | | |

**Table 8**: The relationship of expression of stroma of Cav-1 and stroma of MCT4 in IMPC cases

| | Stroma MCT4 | | | |
|---|---|---|---|---|
| | - | + | ++ | *P |
| Stroma Cav-1 | | | | |
| - | 8(16.3) | 10(20.4) | 31(63.3) | <0.001 |
| + | 0 | 0 | 21(100) | |
| ++ | 8(50) | 1(6.3) | 7(43.7) | |

*Kruskal-Wallis Test.

**Table 9**: The relationship between the expression of Cav-1 and MCT4 and the clinicopathological features of IMPC

| Clinical pathological features | Stroma Cav-1 | | | | Stroma MCT4 | | | |
|---|---|---|---|---|---|---|---|---|
| | - | +/++ | $r_s$ | *P | -/+ | ++ | $r_s$ | *P |
| Age | | | | | | | | |
| ≤52 | 27(61.4) | 17(38.6) | 0.091 | 0.406 | 16(59.3) | 28(47.5) | 0.110 | 0.315 |
| >52 | 22(52.4) | 20(47.6) | | | 11(40.7) | 31(52.5) | | |
| Tumor size (T-stage) | | | | | | | | |
| T1 | 5(35.7) | 9(64.3) | -0.252 | 0.021 | 3(11.5) | 11(19) | -0.044 | 0.694 |
| T2 | 27(54.0) | 23(46.0) | | | 17(65.4) | 33(56.9) | | |
| T3-T4 | 15(75.0) | 5(25.0) | | | 6(23.1) | 14(24.1) | | |
| Histological grade | | | | | | | | |
| I | 1(14.3) | 6(85.7) | -0.178 | 0.101 | 5(18.5) | 2(3.4) | 0..094 | 0.389 |
| II | 32(59.3) | 22(40.7) | | | 14(51.9) | 40(67.8) | | |
| III | 16(64.0) | 9(36.0) | | | 8(29.6) | 17(28.8) | | |
| Lymph nodes staging | | | | | | | | |
| N0 | 5(41.7) | 7(58.3) | -0.223 | 0.040 | 4(15.4) | 8(13.6) | 0.289 | 0.019 |
| N1 | 5(29.4) | 12(70.6) | | | 8(30.8) | 9(15.3) | | |
| N2 | 15(75.0) | 5(25.0) | | | 9(34.6) | 11(18.6) | | |
| N3 | 23(63.9) | 13(36.1) | | | 5(19.2) | 31(52.5) | | |
| ER staging | | | | | | | | |
| Negative | 14(66.7) | 7(33.3) | 0.105 | 0.341 | 4(14.8) | 17(29.3) | -0.156 | 0.153 |
| Positive | 35(56.1) | 29(43.9) | | | 23(85.2) | 41(70.7) | | |
| PR staging | | | | | | | | |
| Negative | 20(69.0) | 9(31.0) | 0.165 | 0.132 | 11(40.7) | 18(31.0) | 0.095 | 0.386 |
| Positive | 29(51.8) | 27(49.2) | | | 16(59.3) | 40(69.0) | | |
| HER-2 Staging | | | | | | | | |
| Negative | 39(56.5) | 30(43.5) | -0.079 | 0.476 | 21(80.8) | 48(82.8) | -0.024 | 0.828 |
| Positive | 10(66.7) | 5(33.3) | | | 5(19.2) | 10(17.2) | | |

*Spearman's Rank-Correlation test.

***Statistical analysis of experimental data***

All the research data was analyzed by SPSS15.0 statistical software, and the P<0.05 as the statistical significance. The Mann-Whitney U test was adopted to compare the clinical pathological data and differences between groups, and also used to compare the expression of MCT4 and Cav-1 in IMPC group and control group of IDC-NOS. Mann-Whitney U test and Spearman rank correlation test were used to compare the expression of Cav-1 and MCT4 intra-class of IMPC group and IDC-NOS group. Spearman rank correlation test was used to analyze the correlation between the Cav-1 and MCT4 in IMPC group and the clinical pathological indexes. Kmskal-Wallis test was used to analyze the relationship between the expression of Cav-1 and MCT4 in IMPC group. Kaplan-Meier was used to draw a survival curve and log-rank test

was used for survival test. Univariate and multivariate survival analyzes were performed using the Cox proportional hazards model.

## RESULTS

### *The clinical pathological features of breast cancer patients*

Tumor size: Among the 86 breast IMPC patients, there were 14 in T1 stage, 50 in T2 stage and 20 in T3-T4 stages; among the 105 IDC-NOS patients, there were 26 in T1 stage, 63 in T2 stage and 16 in T3 stage. There was no statistical significance of the tumor size difference between the IMPC and IDC-NOS (Z=-1.709, P=0.03, see table 2).

Histological grade: there were 7 cases in stage I, 54 in stage II, 25 in stage III among the 86 IMPC patients; there were 6 in stage I, 79 in stage II and 20 in stage III among the 105 IDC-NOS patients. There was no statistical significance of the histological grade between the IMPC group and IDC-NOS group (Z=-1.104, P=0.270, see table 2).

Lymph node staging: among the 85 IMPC breast patients, 12 had no lymph node metastasis, 17 in N1 with lymph node metastasis, 20 in N2 stage and 36 in N3. In the 85 IMPC cases, 73 cases (85.9%) had positive lymph nodes, the number of metastasis was 1-53 and the average was 11.71 ±10.14; in 104 IDC-NOS patients, 38 had no lymph node metastasis, 31 in N1 stage had lymph node metastasis, 15 in N2 stage had lymph node metastasis, 20 in N3 stage had lymph node metastasis. In 104 cases of IDC-NOS, 66 cases (63.4%) had positive lymph nodes, the number of metastasis was 1-48 and the average was 8.79 ±10.31. Compared with IDC-NOS group, IMPC had a higher lymph node staging (Z=-4.515, P<0.001, see table 2).

The expression of ER, PR and HER-2: in 85 IMPC patients, the positive cases of ER and PR were 64 (75.3) cases and 56 (65.9%) cases, respectively; In 84 IMPC patients, the positive cases of HER-2 was 15 (17.9%); The positive cases of ER and PR were 69 (66.3%) and 63 (60.6%) in 104 IDC-NOS patients, respectively; the positive cases of HER-2 was 24 (23.8%) in 101 IDC-NOS patients (see table 2).

### *The expression of Cav-1 in tumor cells and stroma of breast cancer*

Cav-1 was expressed in IMPC tumor cells and tumor stroma CAFs (fig. 5), and the expression in IMPC and IDC-NOS was shown in table 3.

In 86 IMPC breast cancer patients, 49 were negative expressed of Cav-1 stroma and 37 were positive; in 86 IMPC cases, 74 cases showed negative expression of tumor epithelium and 12 showed positive expression. In IMPC group, the positive rate of the expression of Cav-1 in tumor stroma CAFs was higher than the expression in tumor epithelium (43%, 37/86 vs. 14%, 12/86; P<0.001) (table 3).

Cav-1 expressed in tumor epithelium cells and tumor stroma CAFs of breast IDC-NOS (fig. 5). Its expression were as follows: of the 105 breast IDC-NOS cases, 38 cases showed negative expression of stroma Cav-1 and 67 positive expression; of the 105 breast IDC-NOS cases, 91 cases showed negative expression of tumor epithelium Cav-1 and 14 positive expression. In IDC-NOS group, the positive expression of tumor stroma CAFs were higher than its expression in tumor epithelium (63.8%, 67/105 vs. 13.3%, 14/105; P<0.001) (table 4).

The expression loss rate of stroma Cav-1 in IMPC group were higher than that in IDC-NOS group (57%, 49/86 vs. 36.2%, 38/105; P=0.004). While the comparison of the expression of tumor epithelium Cav-1 between the IMPC group and IDC-NOS group suggested that there was no statistical significance (P=0.901) (table 5).

In addition, Cav-1 in normal breast tissue of the ductal and lobular myoepithelial cells are generally expressed, but without expression in the luminal epithelial cells. and there were a small amount expression in the lobules and interstitial fibroblasts around the lobules, while positive expression was also found in adipocytes and endothelial cells (fig. 5A).

### *The expression of MCT4 in breast tumor cells and stroma*

MCT4 expressed in tumor epithelium cells and tumor stroma CAFs in breast IMPC (fig. 6), and its expression in IMPC and IDC-NOS were shown in table 6.

In 86 IMPC cases, 27 cases were negative or low expressed in tumor epithelium of MCT4 and 59 highly expressed; in 86 IMPC cases, 38 cases were negative or low expressed in tumor epithelium cells MCT4 and 48 highly expressed, and there was no obvious correlation of the expression of MCT4 both in tumor epithelium and stroma ($r_s$=0.205, P=0.058) (table 6).

In 105 IDC-NOS cases, 54 cases were negative or low expressed in tumor stroma MCT4 and 51 highly expressed; in 105 IDC-NOS cases, 55 cases were negative or low expressed in tumor epithelium MCT4 and 50 highly expressed, while there was no obvious correlation of the expression of MCT4 both in tumor epithelium and stroma ($r_s$=-0.125, P=0.203) (table 7).

The expression of stroma MCT4 in IMPC group was significantly higher than that in IDC-NOS group (68.6%, 59/86 vs. 48.6%, 51/105; P=0.005). The comparison of

the expression of tumor epithelium Cav-1 between the IMPC group and IDC-NOS group showed that there was no statistical significance (table 5).

***The correlation of the stroma of Cav-1 and MCT4***

The correlation of stroma of Cav-1 and MCT4 was shown in table 8. The loss expression of stroma Cav-1 was related to the high expression of stroma MCT4 (P<0.001).

***The correlation between the expression of stroma Cav-1 and clinical pathological parameters***

The correlation between the expression of stroma Cav-1 and clinical pathological parameters were shown in table 9. The expression of Cav-1 was negatively correlated with tumor size ($r_s$ =-0.252, P=0.021) and lymph node staging ($r_s$ =-0.223 , P=0.040), that were, the bigger the size, the higher the staging of lymph nodes, the higher the loss expression of stroma Cav-1. There was no obvious correlation with the age, histological grade, ER, PR and HER-2 (P>0.05).

***The correlation between the expression of stroma MCT4 and clinical pathological parameters***

The correlation between the expression of Cav-1 and clinical pathological parameters was also shown in table 9. The expression of MCT4 was positively correlated with lymph nodes staging ($r_s$ =0.298, P=0.019), while there was no obvious correlation with the age, tumor size, histological grade, ER, PR and HER-2 (P>0.05).

## DISCUSSION

Tumor cells are not independent survival, but surrounded by a complex microenvironment. In recent years, it has been found that activated fibroblasts in tumor stroma have the same phenotype as wound healing related fibroblasts. These "reactive stromal fibroblasts" and "cancer associated fibroblasts" share the same origin and lineage (Eyden, 2008; Kalluri and Zeisberg, 2006), suggesting that they are derived from genetic or epigenetic transformation. Cav-1 is a scaffold protein on cell membrane, and the mechanism of Cav-1 promoting or inhibiting tumor is not clear at present. The different expression of Cav-1 in different tumor tissues may be associated with the specificity of malignant tumor tissue, the expression of hormones and various growth factors and the mutations of Cav-1 gene itself (Hue-Beauvais *et al*., 2007), but the differences of human operation, observation error and experimental methods during the experimental process cannot be ruled out. A few studies have analyzed the expression of interstitial MCT4 in human breast cancer (Whitaker-Menezes *et al*., 2011; :Choi *et al*., 2013). In our study, 68.6% cases of IMPC were found to be up-regulated in interstitial MCT4, and its up-regulated expression was associated with metastatic lymph node staging and clinical prognosis in MCT4 cases. The expression of phyllodes tumor interstitial MCT4 was detected by immunohistochemistry test by Kwon *et al*, and found that its high expression was related to the disease progression and metastasis (Kwon *et al*., 2013).

## CONCLUSION

Our study confirmed that the loss expression of Cav-1 in tumor stroma CAFs and the up-regulated expression of MCT4, which was related to the poor prognosis of IMPC patients. In further study, the mechanism of interaction between tumor microenvironment and tumor cells may provide a new method for the treatment of breast cancer patients.

## REFERENCES

Cha MJ, Lee HY, Lee KS, Jeong JY, Han J, Shim YM and Hwang HS (2014). Micropapillary and solid subtypes of invasive lung adenocarcinoma: Clinical predictors of histopathology and outcome. *J. Thorac. Cardiovasc. Surg*., **147**(3): 921-928.

Choi J, Kim DH, Jung WH and Koo JS (2013). Metabolic interaction between cancer cells and stromal cells according to breast cancer molecular subtype. *Breast Cancer Res.*, **15**(5): R78.

Eyden B (2008). The myofibroblast: Phenotypic characterization as a prerequisite to understanding its functions in translational medicine. *J. Cell. Mol. Med.*, **12**(1): 22-37.

Fu L (2004). Diagnosis of breast invasive papillary carcinoma. *Chin. J. Pathol.*, **33**(4): 305-307.

Fu L, Song Shan YS, Fu XY, Liu TH and Tu Wu ZY (2004). Relationship between biologic behavior and morphologic features of invasive micro papillary carcinoma of the breast. *Chin Pathol*., **33**(1): 21-25.

Guo X, Chen L, Lang R, Fan Y, Zhang X and Fu L (2006). Invasive micro papillary carcinoma of the breast; association of pathologic features with lymph node metastasis. *Am. J. Clin. Pathol.*, **126**(5): 740-746.

Hue-Beauvais C, Pechoux C, Bouguyon E, Chat S, Truchet S, Pauloin A, Le Gouar Y and Ollivier-Bousquet M (2007). Localisation of aveolin in mammary tissue depends on cell type. *Cell Tissue Res*., **328**(3): 521-536.

Kalluri R and Zeisberg M (2006). Fibroblasts in cancer. *Nat. Rev. Cancer*., **6**(5): 392-401.

Kwon JE, Jung AH and Koo JS (2013). The expression of metabolism-related proteins in phyllodes tumors. *Tumour Biol*., **34**(1): 115-124.

Liu F, Lang R, Wei J, Fan Y, Cui L, Gu F, Guo X, Pringle GA, Zhang X and Fu L (2009). Increased expression of SDF-1/CXCR4 is associated with lymph node metastasis of invasive micro papillary carcinoma of the breast. *Histopathology*, **54**(6): 741-750.

Martinez-Outschoorn UE1, Pavlides S, Whitaker-

Menezes D, Daumer KM, Milliman JN, Chiavarina B, Migneco G, Witkiewicz AK, Martinez-Cantarin MP, Flomenberg N, Howell A, Pestell RG, Lisanti MP and Sotgia F (2010). Tumor cells induce the cancer associated fibroblast phenotype via caveolin-1 degradation: implications for breast cancer and DCIS therapy with autophagy inhibitors. *Cell Cycle*, **9**(12): 2423-2433.

Nam KH, Lee BL, Park JH, Kim J, Han N, Lee HE, Kim MA, Lee HS and Kim WH (2013). Caveolin 1 expression correlates with poor prognosis and focal adhesion kinase expression in gastric cancer. *Pathobiology*, **80**(2): 87-94.

Reis-Filho JS and Ellis IO (2012). WHO classification of tumours of the breast. *Lyon: lARC Press WHO*: 65-66.

Tavassaoeli FA and Davilee P (2003). Pathology and Genetics of Tumours of the Breast and Female Genital Organs. *Lyon: lARCPress WHO*: pp.28-29.

Whitaker-Menezes D, Martinez-Outschoorn UE, Lin Z, Ertel A, Flomenberg N, Witkiewicz AK, Birbe RC, Howell A, Pavlides S, Gandara R, Pestell RG, Sotgia F, Philp NJ and Lisanti MP (2011). Evidence for a stromal-epithelial "lactate shuttle" in human tumors: MCT4 is a maker of oxidative stress in cancer-associated fibroblasts. *Cell Cycle*, **10**(11): 1772-1783.