The study of fatty acid binding protein expression in human breast cancer tissue and its significance

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ABSTRACT: Purpose: To detect the expression pattern of Fatty Acid Binding Proteins (FABPs) in human breast cancer and try to find potential markers and therapeutic targets for breast cancer. Methods and materials: Adipocyte- FABP (A-FABP), Heart or Muscle FABP (H-FABP), Brain-FABP (B-FABP), Epidermis or psoriasis FABP (E-FABP), Liver-FABP (L-FABP), Intestinal FABP (I-FABP) and Gastro- FABP (G-FABP) expression diversity were detected by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR), immunohistochemistry staining and Western Blot analysis in 41 ductal infiltrating carcinoma and 17 fibroadenoma of breast. Results: From RT-PCR, immunohistochemistry and Western Blot studies, it was found that there was no significant difference between ductal infiltrating carcinoma and fibroadenoma in the expression of A-, B-, G-, and I-FABP (p>0.05), while E, H-and L-FABP were elevated significantly in ductal infiltrating carcinoma (p<0.05) compared with fibroadenoma. Conclusion: E-, L-FABP and H-FABP play an important role in the development of invasive ductal breast cancer. This study provides a theoretical basis to further explore the molecular markers and therapeutic approach of invasive ductal breast cancer.

KEYWORDS: gene expression, Fatty Acid binding expression, ductal infiltrating carcinoma, breast

Fatty acid binding proteins (FABPs) are small molecule proteins that mediate lipid droplets transportation and utilization, as well as participate in fatty acid metabolism [1-2]. Seven kinds of fatty acid binding proteins have been isolated so far, and they are named according to their sources: Adipocyte-FABP (A-FABP) [3], Brain-FABP (B-FABP) [4], Epidermis or Psoriasis FABP (E-FABP) [5], Gastro- FABP (G-FABP) [6], Heart or Muscle FABP (H-FABP) [7], Intestinal FABP (I-FABP) [8] and Liver-FABP (L-FABP) [9].

In recent years, it was found that FABPs control cancer growth through combination with the fatty acid transporters and their derivatives [1], carcinogens [2], hormones [10], steroids [11] and other materials. Different FABPs have different roles on cell growth [12]. L-FABP transfection in hepatoma cells can promote cancer cell proliferation [13]. Addition of L-FABP oligodeoxynucleotides to breast cancer cell line MCF-7 and T47D[14] or prostate cancer cell line DU145 [15] results in cell proliferation-related genes downregulated. On the contrary, H-FABP transfection in the breast cancer cell line MCF-7 inhibits cell growth[16], leading to the loss of tumor characteristics; A-FABP Transfection in prostate cancer cell line DU145 results in apoptosis[17].

However, there is still a lot of controversy on the exact roles of FABPs on tumors. It is still not clear about the expression pattern of FABP family members in human breast cancer tissue and their role and significance on the development of breast cancer.

Therefore, in this article semi-quantitative RT-PCR, immunohistochemistry, Western Blot and etc were used to observe the
FABPs expression in 35 cases of invasive ductal breast carcinoma through comparison with fibroadenoma, in order to explore the role and molecular mechanism of FABPs in the progress of invasive ductal carcinoma.

Materials and Methods

Material

From May 2005 to May 2011 we collected 58 cases of breast specimens (41 cases of invasive ductal carcinoma, 17 cases of fibroadenoma, age 38 to 65) at Breast Surgery of West China Hospital of Sichuan University. With patients’ consent, the specimens were drawn in operation without preoperative chemotherapy or radiotherapy of patients. The specimens were immediately placed in liquid nitrogen and stored. Each sample was H.E. stained.

RT-PCR detection

The total RNA extraction and reverse transcription of cDNA were referenced to the steps from RNA extraction kit (Shanghai Hua Shun) and Revert AidTM first strand cDNA synthesis kit (forements), respectively. Primers designed and synthesized by Takara were used for the PCR amplification of target fragments (adipocyte-FABP, heart or muscle FABP, brain-FABP, epidermis or psoriasis FABP, liver-FABP, intestinal FABP and gastro-FABP). The primer sequences are shown in Table 1.

The number of semi-quantitative RT-PCR cycles of the target gene was 28. When β-actin was used as an internal reference, the number of cycles was 26. For different samples a UV spectrophotometer (Eppendorf) was used to determine the cDNA concentrations. The same amount of cDNA was used for each PCR reaction. A 25 μl reaction system contained 1 μl cDNA template, 12.5 μl PCR master mix (Takara) and 1 μl each of the target gene upstream and downstream primers (0.5 μmol / L). The PCR products were observed and recorded under 1.5% agarose (Sigma) gel electrophoresis (voltage 70 – 100mv) and gel imaging system (Boi-Rad). The mRNA expression change was measured by the optical density ratio between the target band by Quantity One program (Bio-Rad, Hercules, CA) and marker1 (ferments) 400bp band.

Immunohistochemical staining

Anti-L-FABP (monoclonal mouse anti-human, MAB2964), H-FABP (monoclonal rat anti-human, MAB1678), and E-FABP (polyclonal goat anti-human, AF3077) antibodies were purchased from R & D systems, Inc. Peroxidase labeled streptavidin staining kit SA1025 was purchased from Wuhan Boster Biological Engineering Co., Ltd. Kit SP-9002 and SP-9003 were supplied by Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. APES (Beijing Zhongshan) processed the slide and conventional dewaxing of paraffin sections to water. The staining procedure was referenced to DAB kit (Beijing Zhongshan).

The results were comprehensively evaluated according to the percentage of positive tumor cells. Five fields were randomly selected under the lens with 100-time magnification, and for each field of vision, 100 cells was counted continuously under 400 mag

<table>
<thead>
<tr>
<th>cDNA</th>
<th>Reference sequence no.</th>
<th>orientation</th>
<th>Annealing temperature</th>
<th>Sequences</th>
<th>Product size</th>
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<td>NM001443</td>
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<tr>
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<td>NM000130</td>
<td>Reverse</td>
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<td>5′GACTTTCCTCCCACGAGTACGAC3′</td>
<td>227</td>
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<tr>
<td>H-FABP</td>
<td>NM004302</td>
<td>Forward</td>
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<td>5′TGGACGACCATGGAAAGTTAGA3′</td>
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<tr>
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<tr>
<td>β-Actin</td>
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<th>Genes</th>
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<th>Percentage of positive cells (M vs B)</th>
<th>IOD value of protein bands (M vs B)</th>
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<td>2.786±0.213/0.742±0.087</td>
<td>0.721±0.048/0.229±0.021</td>
<td>0.593±0.041/0.115±0.015</td>
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<tr>
<td>L-FABP</td>
<td>1.56</td>
<td></td>
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<tr>
<td>H-FABP</td>
<td>1.425±0.175/0.457±0.136</td>
<td>0.589±0.041/0.115±0.015</td>
<td>0.819±0.027/0.178±0.036</td>
</tr>
</tbody>
</table>

Tab. 1 RT-PCR primer sequences for 7 FABPs genes

Tab. 2 Different expression of E-, L- and H-FABP between malignant(M) and benign(B) breast tissues (x±s)
nification. The number of positive cells was recorded to calculate the percentage, and the average figure was taken.

**Western Blot analysis**

50-100mg tissue was grinded to fineness under liquid nitrogen. 1ml Lysate [7M urea (Sigma, USA), 4% CHAPS (Bio-Rad, USA), 40mM DTT (Amerasco, USA), 100mM PMSF, (Amerasco, USA), 0.05mg/ml RNA enzyme, 0.2mg/ml DNA enzyme, 0.001% bromophenol blue] was mixed well with the tissue, and stored at 4 °C for 15 minutes. The mixture was centrifuged at 4 °C for 40 minutes (16000 × g), and the supernatant was collected.

The content of each sample was determined by Bio-Rad spectrophotometer with reference to steps from Pierce protein content assay kit.

1:1 Ratio of the sample and 1 × SDS (Amerasco, USA) sample buffer (5 × SDS sample buffer: 0.5MTris-HCL (Amerasco, USA), pH 6.8.2.5 ml; 7.8% DTT, 10% SDS, 0.005% bromophenol blue, glycerol 2.5ml) was mixed well and denatured at 100 °C for 5 minutes. Each well was pipetted 30ug. Protein bands were separated at 4% stacking gel and 12% separating gel under 80V. The time for semi-dry transfer under 200mA was 25 minutes (6.5cm × 8.5cm PVDF membrane, Millipore).

The membrane was incubated at 4 °C overnight with an anti-E-, H-, L-FABP and Actine (Santa Cruz) respectively. It was incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz, Beijing Zhongshan-packing ZB-2301, ZB-2306 -2 305) at room temperature for 2h.

The target band was detected with enhanced chemical luminescence Kit (Pierce). The optical density of band was determined with Quantity One program (Bio-Rad, Hercules, CA).

**Statistical analysis**

SPSS software (version 13) was used for statistical analysis, and t test was applied to determine the difference between the two groups. If P <0.05, it is considered a significant difference.

**Results and Discussions**

**FABP mRNA expression in the two breast tissues**

A-B-, E-, H-, I-, L-FABPs were detected in both fibroadenoma and invasive ductal breast cancer.

The statistical analysis results showed that compared with fibroadenoma, E-, H- and L-FABP mRNA in invasive ductal carcinoma were notably increased (P <0.05, table 2, figure 1). There was no significant difference between the two tissues in the expression of B-, E-, G-, I-FABP mRNA.

**Immunohistochemical staining of in situ observation**

H-FABP positive cells in both fibroadenoma and invasive ductal carcinoma were distributed in the interstitial cells (Figure 2). E-FABP positive cells in fibroadenoma were confined to vascular endothelial cells; in invasive ductal carcinoma the positive cells were not only in vascular endothelial cells, but also were distributed in the interstitial cells, acinar and ductal epithelial cells (Figure 3).

L-FABP positive cells in fibroadenoma and invasive ductal carcinoma were in the epithelial cells of acinar and catheter (Figure 4).
4).

The statistical results showed that the percentages of positive cells of E-, H- and L-FABP in invasive ductal breast cancer tissue were significantly higher than those in fibroadenoma.

**Western Bolt analysis results**

The results of semi-quantitative Western Blotting showed that E-, H- and L-FABP expressions in invasive ductal carcinoma were significantly elevated compared with fibroadenoma (p <0.05) (Figure 5).

The results showed that the expressions of E-, H- and L-FABP at the protein level and mRNA level were basically the same.

This study was the first quantitative and positioning research in the expression of E-FABP mRNA and protein in infiltrating ductal carcinoma. The results showed that the expressions of E-, H- and L-FABP in invasive ductal carcinoma increased significantly compared with benign fibroadenoma. We speculated that they are related to the occurrence and pathological process of invasive ductal carcinoma.

**Expression of E-FABP in invasive ductal breast cancer**

E-FABPs are small molecule proteins distributed in skeletal muscle and placenta microvascular[18]. Because of their over expression in psoriasis, they are also known as psoriasis FABP. Recently, it was found that E-FABP showed over-expression in the resistant cell lines of skin cancer, pancreatic cancer[19] and colon cancer[20]. Recently, it was found that E-FABP was a potential prognostic marker and target for tumourigenicity-suppression[21]. In addition, studies further confirmed that E-FABP is a target gene of c-myc, and it increases rapidly when induced by EpCAM [22]. The over expression of EpCAM is closely related to epithelial cell differentiation and a high degree of proliferation. Therefore, EpCAM and c-myc may be used as the upstream factor of the E-FABP. At specific stages, they can turn on and off the E-FABP expression and regulate tumor cell proliferation and differentiation. However, Das found that E-FABP expression in breast cancer cell line MCF-7, T47D and prostate cancer cell line DU145 was significantly lower than normal cell lines. Therefore, the role of E-FABP on tumor remains controversial, and there was no report on the E-FABP expression in breast cancer. Our results showed that compared with breast fibroadenoma, E-FABP expression in invasive ductal carcinoma was stronger and had wider distribution, suggesting that E-FABP is closely related to the development of tumor cells, but its mechanism of action is to be further explored.

**Expression of H-FABP in invasive ductal breast cancer**

Because H-FABP can suppress proliferation and promote differentiation on the developing mammary gland and skeletal muscle cell [23], it was considered a tumor suppressor gene. Later it was found that transfected or knockout gene H-FABP had no notable effects on mouse mammary cell proliferation and differentiation. Therefore, the exact role of H-FABP in the mammary epithelial is unclear. The results here showed that H-FABP level in invasive ductal carcinoma compared with fibroadenoma was significantly enhanced. This further demonstrated that H-FABP as a tumor suppressor gene is not entirely valid. The possible reasons are as follows: First, the inhibition of H-FABP on breast cancer cells may be associated with point mutations. Studies have shown that while the recombinant H-FABP has no inhibition on the growth of Ehrlich ascites tumor cell and gastric cancer, mutant H-FABP (threonine 127 replaced by valine or the C-terminal alanine replaced by the stop codon) can inhibit the growth of cultured cells [24]. Second, the inhibition of H-FABP on breast cancer may be related to tumor-associated signaling system. Studies have shown that peptides P108 derived from H-FABP inhibit EGF cloned breast cancer cell line MDA-MB486, which indicates H-FABP may suppress breast cancer by affecting the EGFR signaling system.

Due to the lower postoperative survival rate (p = 0.0004) of H-FABP-positive patients than that of negative patients, it is considered to be a molecular marker of poor prognosis for gastric cancer. However, further study is needed to determine the relationship of H-FABP and postoperative survival rate of invasive ductal carcinoma patients.

**Expression of L-FABP in invasive ductal breast cancer**

A large number of experiments have shown that L-FABP can promote tumor cell proliferation and inhibit differentiation. L-FABP has higher expression in lung cancer [25], small bowel tumors, and gastric adenocarcinoma [26]. In breast cancer cell lines MCF-7, T47D and the prostate cell line DU145 L-FABP expression is significantly up regulated compared with normal cell lines. In addition, liver cells transfected with L-FABP obtain proliferative ability. The present study demonstrated that L-FABP expression was significantly higher in invasive ductal breast cancer tissue than in fibroadenoma. This is further evidence that L-FABP is closely related to the occurrence of invasive ductal carcinoma.

Fatty acid binding proteins are a key factor for fatty acid trans-
port, storage and energy metabolism in the breast tissue, very similar to their function in normal cells. However, in tumor cells, FABPs can not only transfer long-chain fatty acids to provide the energy and raw materials for cancer growth, but also can combine and transfer fatty acid derivatives, hormones, steroids and carcinogens as the first or second messenger to activate or inhibit signaling pathways related to tumor progress, so they can regulate tumor cell proliferation, survival, and metastasis. In this study, RT-PCR method was used to detect the expression diversity of seven kinds of FABPs mRNA in 51 cases of breast tissue. E-, H-, L-FABPs genes with significant difference between benign and malignant breast tissue were screened out, followed by the observation of protein localization and quantification. The results showed that the E-, H-, L-FABP expressions in invasive breast cancer compared to fibroadenomas were significantly up regulated, which indicated that the fatty acid binding proteins were tightly associated with invasive ductal breast cancer development. However, the mechanism of action is unclear. This study provided basic data and theoretical basis to further explore the molecular markers and therapeutic approach to invasive ductal breast cancer.

Acknowledgments

This work was supported by the fund of the horizontal collaboration project (No. 10H1324); The authors gratefully acknowledge the support of the West China Hospital Breast Surgery and Chengdu Medical College.

REFERENCES

