Int J Clin Exp Pathol 2017;10(7):7743-7749
www.ijcep.com /ISSN:1936-2625/IJCEP0055254

Original Article

Differential distribution of immune cells in breast invasive carcinoma vs. breast carcinoma *in situ* and its significance in interpretation of immune surveillance

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Received April 12, 2017; Accepted June 23, 2017; Epub July 1, 2017; Published July 15, 2017

Abstract: Immune surveillance is a highly controversial subject in both the field of immunology and cancer biology. On one hand, in spite of extensive studies, there is no cancer specific antigens identified. Yet, the organisms do exert immune response to tumors. On the other hand, it is believed that immune surveillance suppresses tumorigenesis by eradicating mutated cells. However, it is also widely known that tumorigenesis is promoted by inflammation, which is in nature immune reaction. In the present study, we tried to find immune cells in early tumor lesions for the supportive or negative evidence of immune surveillance. We used immunohistochemistry to observe the localization and distribution of immune cells in the in situ carcinoma lesions and in the invasive cancer of breast. Interestingly, we did not see immune cells in either ductal carcinoma in situ (DCIS) or lobular carcinoma in situ (LCIS) of breast, the two basic supposed early cancer forms. In contrast, we observed extensive infiltration of immune cells in the invasive breast cancer, and close contact between immune cells and tumor cells. Based on these findings, we propose that the tumor antigens of breast cancer are not derived from the gene mutation or amplification such as HER2, but rather from misplacement of epithelial cells in the mesenchymal tissue. To avoid being targeted by the immune system, the carcinoma cells exert epithelial-mesenchymal transition (EMT). Therefore, immunosurveillance could be regarded as preventing the intrusion of epithelial cells to mesenchymal tissues, and EMT is a form of immune escape by the strategy of mimicry.

Keywords: Immunosurveillance, breast cancer, basement membrane, carcinoma in situ, epithelial mesenchymal transition

Introduction

Carcinogenesis has been widely accepted as a result of accumulated gene mutations [1]. But numerous paradoxes arouse from this hypothesis [2-6]. One issue is that the mutation rate is rather high, but cancer is relatively rare. Taken the mutation rate as 10^{-6}, a cell would get 10^3 mutations in one division. If the correction mechanism repairs 99% of the DNA replication errors, there still would be around 10 mutations left in a newly produced cell. The explanation for the discrepancy of gene mutation rate and the cancer incidence is generally attributed to two factors, the neutral mutation [7] and the immunosurveillance [8, 9]. Neutral mutation theory holds that most of the mutations are harmless and have no biological effect on the cells, and the immunosurveillance theory posits that the transformed cells from harmful mutations are eradicated by the immune systems. Yet the immunosurveillance is another highly controversial theme [10].

Since 1960s the concept of immunosurveillance has undergone four distinct eras of acceptance/abandonment. These include a general acceptance during 1957-1974, an abandonment during 1974-1996, and a resurrection during 1996-2001 in the form of an ele-

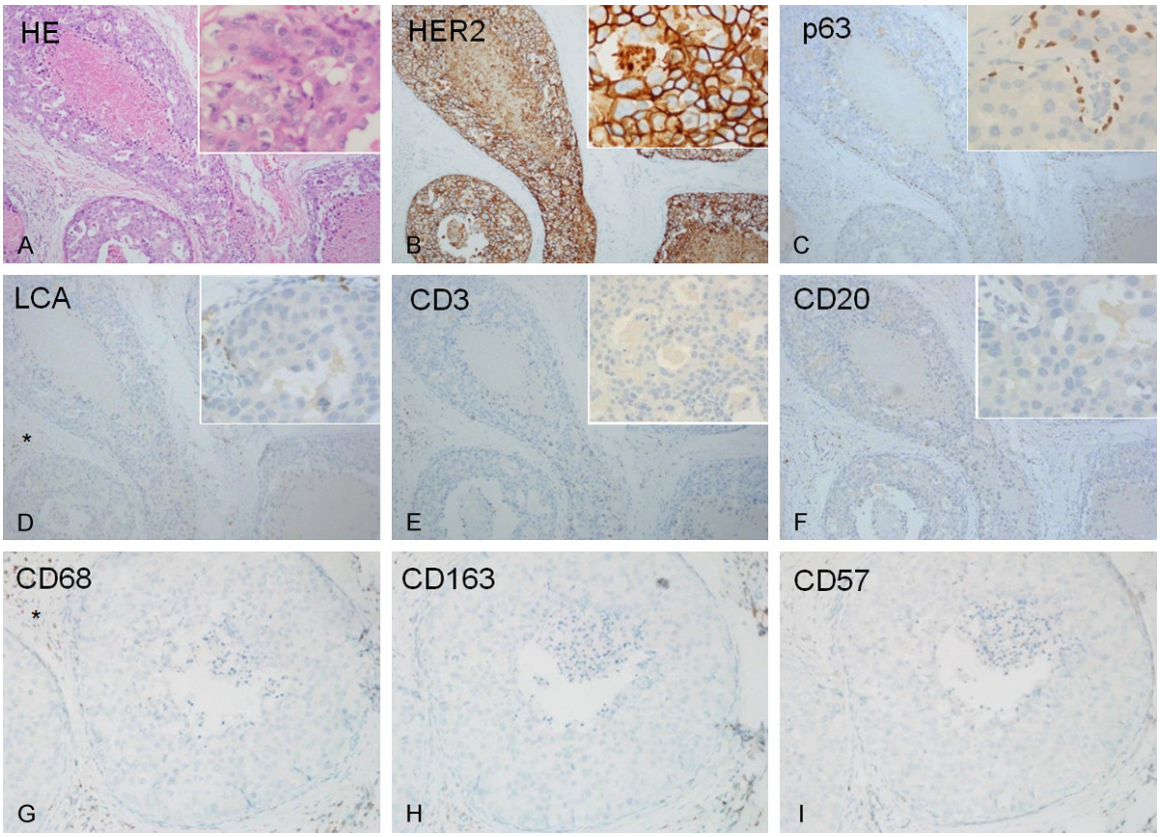

Figure 1. Immunohistochemistry revealing the immune cells in DCIS of breast. Immune cells cannot be seen inside the DCIS lesion of breast. Consecutive sections were stained for various markers. P63 was stained to reveal the existence of myoepithelial cells, a confirmative marker of DCIS. In these two cases of HER2 positive DCIS, no immune cells were seen inside the DCIS lesion. LCA, leukocyte common antigen; CD20, for B cells; CD3 for T cells; CD68 and CD163 for macrophages; CD57 for NK cells. There are immune cells stained in the stroma (*), which can be used as control. (A-F) is from one case, and (G-I) is from a separate case which is also HER2 positive.

gant theory of tumor immunoediting proposed by Robert Schreiber et al, and a retreat since 2006. Tumor immunoediting is divided into three stages, namely immune clearance, immune tolerance and immune escape [10]. Immunoediting theory has attracted wide attention and many related studies have been conducted on it. However, critical paradoxes still remain non-elucidated. The first issue is about the cancer antigens. For most types of cancer, there are no specific antigens identified. Therefore, it is hard to understand what the immune reactions target at. The second paradoxical issue is the morphological evidence of immunosurveillance. Technically, immunosurveillance requires the presence of immune cells in the spot of cell proliferation where gene mutation happens. But the presence of these infiltrative immune cells would be defined as inflammation, which is now generally accepted as a strong cancer promoting factor.

Literally, imunosurveillance means a close watch of those cells that might go awry and then eradicate it before it spread further. By this sense, many people suspect its real existence. Even Burnet who proposed this concept admitted that it was an issue hard to prove [9]. We here tried to search for the existence of immune reactive cells inside the supposed early form of cancer--carcinoma *in situ*, to elucidate if the immune system keeps a surveillance of gene mutations in the epithelial cells or not.

Materials and methods

Tissue samples and antibodies

Altogether 23 cases of ductal carcinoma *in situ* (DCIS) with invasive breast cancer, 7 cases of lobular carcinoma *in situ* (LCIS) with invasive lobular carcinoma were selected for immunohistochemical studies. All the specimens were

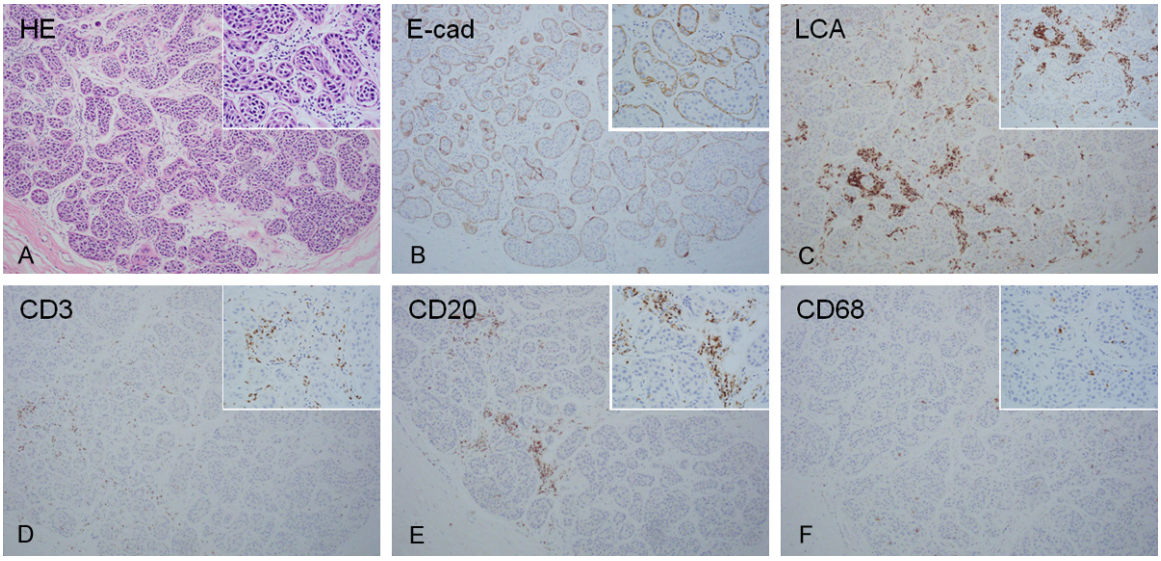

Figure 2. Immunohistochemistry revealing the immune cells in LCIS of breast. A. HE staining shows the histology of the lesion. B. E-cadherin was stained to reveal the intercellular connections. The myoepithelial cells were positively stained but the luminal cells were all negative, which is a hallmark of LCIS. C-F. LCA, CD3, CD20 and CD68 were stained to reveal total and different types of immune cells. Note that there are not immune cells seen inside the LCIS lesion, but abundantly exist in the stroma.

surgically removed and the patients were not subjected to presurgical chemical treatment or radiation treatment. In addition, 10 cases of cervical intraepithelial neoplasia (CIN) were also used for immunohistochemical studies. All the antibodies were purchased from MXB of Fuzhou, China.

Immunohistochemistry

All the archived samples were fixed with neutral buffered 10% formalin and routine paraffin sections were cut and mounted on positively charged glass slides. Immunohistochemistry procedures were performed by using Ventana BenchMark GX automated staining machines. The staining was revealed with DAB-H_2O_2 and counterstained with hematoxylin.

Periodic acid-Schiff staining

PAS staining were done with standard procedures. Briefly, the sections were deparaffinized and rehydrated to water, and then oxidized in 0.5% periodic acid solution for 5 minutes followed by a brief wash with distilled water. Next the sections were placed in Schiff reagent for 15 minutes followed by a wash in tap water for 5 minutes. Finally, the sections were counterstained with Mayer's hematoxylin and mounted for examination.

Results

Existence of immune cells in breast invasive carcinoma vs. carcinomas in situ

There are two forms of carcinoma *in situ* in the breast pathology, ductal carcinoma *in situ* (DCIS) and lobular carcinoma *in situ* (LCIS) [11]. DCIS is the most frequently diagnosed *in situ* cancer of breast and is believed to be the precursor lesion of invasive breast cancer (IBC) [11, 12]. Interestingly, More than 50% of DCIS lesions have HER2 oncogene amplification and overexpression which was believed by many to be a target of cancer immune response [13]. Cancer genome studies also revealed a similar pattern of gene signatures in DCIS with that in IBCs [14]. In an effort to find morphological evidence for immunosurveillance, we examined the existence of immune cells in DCIS, LCIS, and IBC by immunohistochemical staining with specific markers of different immune cells. The antibodies we used included leukocyte common antigen (LCA) for all the immune cells, CD3 for T lymphocytes, CD20 for B lymphocytes, CD57 for NK cells, both CD68 and CD163 for macrophages.

Unfortunately, we failed to find any leukocytes in the lesions of both 23 cases of DCIS including 12 cases with HER2 overexpression, and 7

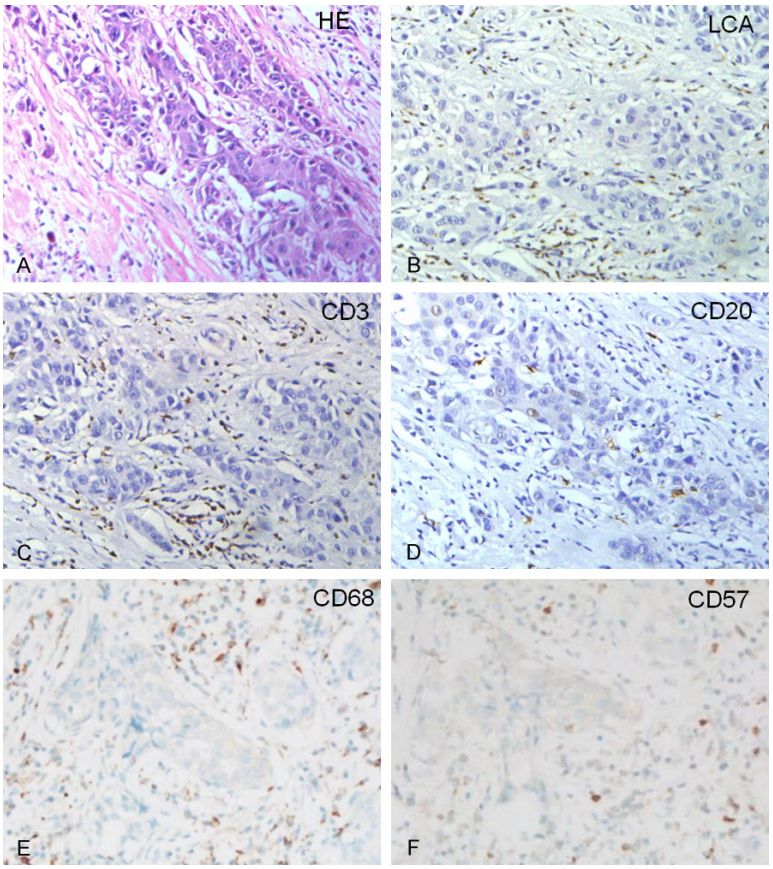

Figure 3. Immunohistochemistry revealing the immune cells in invasive ductal carcinoma of breast. A. HE staining shows the histology. Small, round lymphocytes are seen in close contact with cancer cells. B-F. Are immunohistochemical staining with different markers for immune cells. LCA, leucocyte common antigen; CD3 for T cells, CD20 for B cells, CD68 for macrophages, and CD57 for NK cells.

cases of LCIS, (**Figures 1**, **2**). Although in some areas of LCIS, CD57 positive staining cells could be seen, they were obviously not NK cells since they did not express LCA (data not shown). Indeed, CD57 has been reported to be expressed nonspecifically in cancer cells [15]. In contrast, lymphocytes are found abundantly exist in the surroundings of DCIS, and LCIS. In the IBCs, however, many immune cells were found infiltrated inside the cancer lesion (**Figures 3**, **4**). These results suggest that both HER2 overexpression and other gene mutations were not the antigens attracting the immune cells.

Damage of basement membrane in cervicitis

Cervical carcinoma is caused by human papilloma virus (HPV) infection. The early lesion of cervical carcinoma is thought to be the cervical intraepithelial neoplasia (CIN). CIN is classified as three degrees, CIN I, CIN II, and CIN III. To check the existence of immune cells in the CIN lesions, we did immunohistochemical staining.

Not surprisingly, we found extensive distribution of lymphocytes infiltrating the CIN lesions, from CIN I, to CIN III (**Figure 5A**, **5B**).

The extensive infiltration of lymphocytes/inflammatory cells definitely indicates inflammation, which is a known factor of carcinogenesis [16]. To see if the inflammatory reactions could have any destruction to the basement membrane (BM), a thin structure separating the epithelium from the connective tissue stroma, we did PAS staining. In many places, we found that the BM was obviously damaged (**Figure 5C-F**), which provided a chance for the immune cell infiltration, and also broke the microenvironment of epithelium.

Discussion

Immunosurveillance is an important part of both the current immunology theory and cancer theory. To find morphological evidence of immunosurveillance, we tried to look for the existence of immune cells in the early lesions of carcinogenesis by immunohistochemical labeling. Unfortunately, we found no immune cells in the two histo-types of *in situ* carcinoma of breast, DCIS and LCIS, no matter with HER2 overexpression and gene amplifications or not. In contrast, abundant immune cells were observed in invasive breast cancer. We also found immune cells in the cervical intraepithelial neoplastic lesions of uterus cervix, which were with damaged basement membrane. These indicate that gene mutations or amplifications are not the target of immunosurveillance, which is a concept we should look for an alternative explanation.

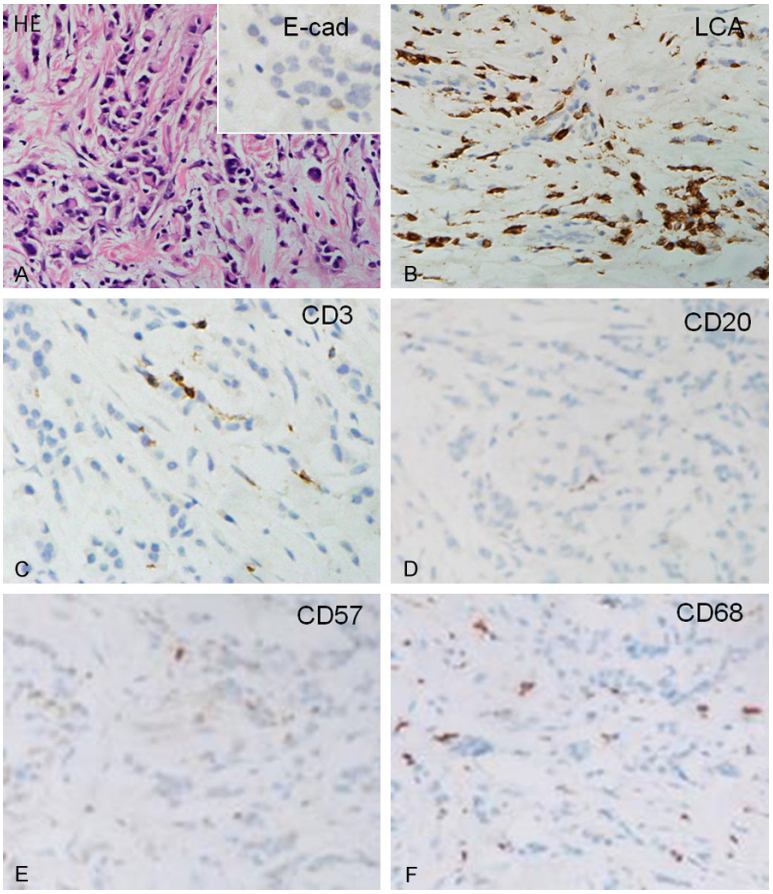

Figure 4. Immunohistochemistry revealing the immune cells in invasive lobular carcinoma of breast. A. HE staining shows the histology. Cancer cells show a loose connection to each other. Inserts shows negative staining of e-cadherin. B-F. Are immunohistochemical staining with different markers for immune cells. LCA, leucocyte common antigen; CD3 for T cells, and CD20 for B cells, CD68 for macrophages, and CD57 for NK cells.

The question further goes to the nature of tumor antigen. So far no tumor specific antigens have been identified for most types of human cancer. But the immune response against cancer definitely exists, as we observed the existence of immune cells surrounding the invasive cancer cells in the present study. For most of instances, gene mutations do not account for the immunogenicity because gene mutations are not less found in benign tumors and *in situ* carcinoma [14, 17]. Then what are probably the immunogens of the immune response against cancer?

To address these paradoxes, we have proposed a novel explanation for the immunogenicity of cancer. In humans, more than 80% of human malignancies are epithelially derived carcinomas. The nature of carcinoma is malignantly transformed epithelial cells inside the mesenchymal tissue. In normal tissues, the epithelium is separated from the mesenchymal tissue by the basement membrane. Furthermore, there are not blood vessels, nor lymphatics inside the epithelium. In contrast, the mesenchymal connective tissue is quite rich in blood vessels, lymphatics, and immune cells. Then the question arise that if the epithelial cells are dislodged into the connective tissue, as in the situation of inflammation which may damage the basement membrane, would it trigger an immune response? In our newly proposed model of carcinogenesis, the invasive cancers are not derived from preformed in situ carcinoma [18, 19]. Instead, they grow out *de novo* from misplaced epithelial stem cells [18, 19].

By the original clonal selection theory of Burnet, T or B cells which recognize their own cells of host were eliminated during embryonic development. So there should be no immune cells which react with its own epithelial cells wherever these cells are located. However, the clonal selection theory has been facing serious challenges for the past decades [20, 21]. For example, it cannot explain the self-antibodies produced in autoimmune diseases [20]. By the novel explanation of the central immune tolerance that the reticular epithelial cells of thymus express a variety of self-antigens which inhibits the T cell clones that might react with its own tissues or cells [22]. We do not know yet if these epithelial cells of thymus express the epithelial marker antigens or not. It is reasonable that they do not, since normally the epithelial cells are separated from the immune system by the basement membrane. Furthermore, by the danger model of Matzinger et al [21], we would believe that immune cells should exert a reaction against the epithelial cells invaded in mesenchymal tissues, whether they have gene mutations or not, since these invaded cells constitute a

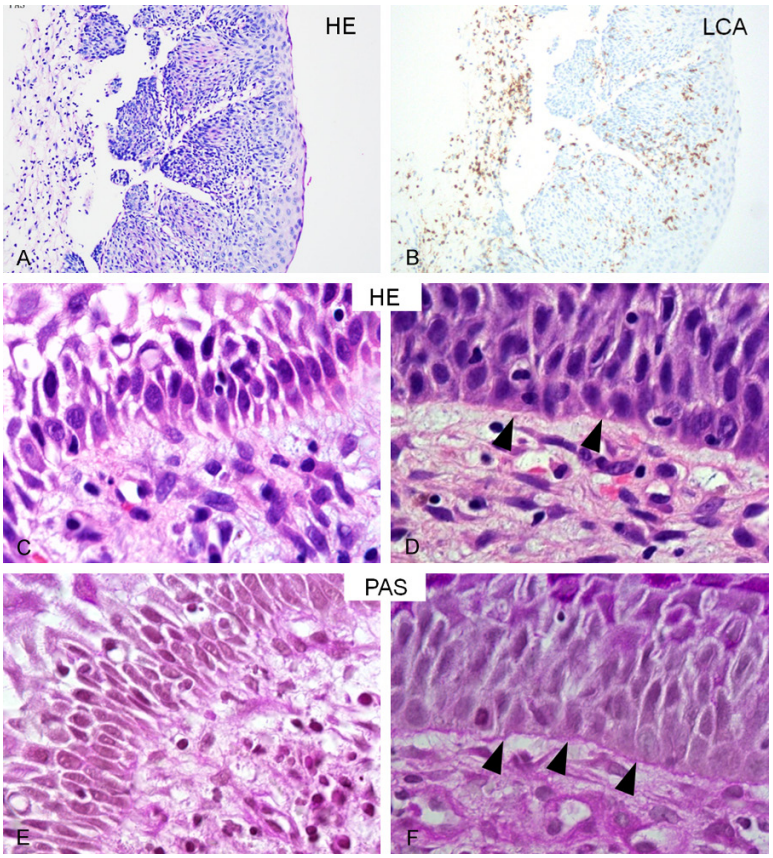

Figure 5. Leucocyte infiltration in CIN lesions and damage of epithelial basement membrane. (A) HE staining to show the histology of CIN lesion. (B) In a consecutive section of (A), immunohistochemical staining of LCA was done to reveal the existence of immune cells. (C-F) High power image with HE and PAS staining to show the damage of BM in CIN. (C and E) Are consecutive sections. The basement membrane was obviously damaged. (D and F) Are normal regions which were used to serve as control. Arrow heads points to the basement membrane.

danger to the organism [23]. By common sense, a responsible house-keeping system would not neglect these intruded epithelial cells inside the mesenchymal tissue. The antigens which the immune cells react against could be just the epithelial specific antigens which distinguish these cancer cells from surroundings.

Epithelial mesenchymal (EMT) transition has been a hot theme of cancer research for the last decade. It has been generally agreed that EMT was a gene regulated process, and an important step for metastasis of carcinoma cells. The molecular hallmark of EMT is the loss of epithelial cell markers such as E-cadherin and the abnormal expression of mesenchymal cell marker such as vimentin [24, 25]. In the mammary gland tumors, the LCIS and the invasive lobular carcinoma are both characterized by the loss of E-cadherin expression. Paradoxically, LCIS has been clinically proved do not further progress [11, 26], which leaves the origin of invasive lobular carcinoma an enigma, and further indicates that the purpose of EMT may not just be to metastasize.

By the above analysis, we understand that the nature of invasive carcinoma cells are epithelial cells invaded/misplaced in the mesenchymal tissues [18, 19]. The epithelial markers could be the target of the immune cells, which may account for the so-called immunosurveillance [27]. The *in situ* carcinoma cells, which are located inside the epithelium and are separated from the mesenchymal tissues by the basement membrane do not constitute a danger to the organism, and therefore, do not elicit immune response, despite that they may have gene mutations or amplifications such as HER2. To avoid being targeted, a smart tacit of cancer cells is to downregulate its expression of the epithelial markers, to assimilate with the surroundings. In fact, this is a universal strategy of any living creature would adapt to increase its chance of survival, no matter as predator or avoiding prey. That is called, mimicry [27].

Acknowledgements

This work was supported by the National Natural Science Foundation of China (30971535) and Shenzhen Science and Technology Innovation Committee (JCYJ20170307143804397) to RA Wang.

Disclosure of conflict of interest

None.

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