Pathol. Oncol. Res. (2014) 20:619–624
DOI 10.1007/s12253-013-9740-z

RESEARCH

# Tumor-Associated Macrophages are Correlated with Tamoxifen Resistance in the Postmenopausal Breast Cancer Patients

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Received: 25 August 2013 / Accepted: 12 December 2013 / Published online: 12 January 2014


**Abstract** Tumor-associated macrophages (TAMs) have been correlated with increased angiogenesis and poor prognosis in breast cancer. However, the precise role of TAMs in tamoxifen resistance remains unclear. We used immunohistochemical method to examine the expression of epidermal growth factor receptor (EGFR) and CD163+ macrophages in 100 breast cancer tissues. The clinical and biological features of 100 patients were estrogen receptor (ER)-positive and human epidermal growth factor receptor 2(Her-2)-negative tumors. The tamoxifen resistant tissues ($n$=48) were the surgical excision samples from patients who developed recurrence or metastasis at the time of adjuvant tamoxifen treatment. The tamoxifen resistant tissues were contrast to tamoxifen sensitive tissues ($n$=52). Positive staining for EGFR and CD163+ macrophages were observed in 21 samples (43.8 %) and in 26 samples (54.2 %) respectively in tamoxifen resistance group, which were higher than that of tamoxifen sensitive group ($P$=0.001 and $P$=0.000279 respectively). Significant positive correlations were found between the expression of EGFR and CD163+ macrophages ($r$=0.567, $P$<0.01). CD163+ macrophages were positively correlated with tumor size, lymph node metastasis and obesity. Obesity was also related to tamoxifen resistance ($P$<0.05). The patients with higher density of CD163+ macrophages infiltration suffered from shorter time to develop recurrence or metastasis ($P$<0.05). TAMs may be associated with tamoxifen resistance. Further studies are needed to investigate the potential mechanism between TAMs and tamoxifen resistance.

**Keywords** Breast cancer · Tumor-associated macrophages · Epidermal growth factor receptor · Tamoxifen resistance · Obesity

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## Introduction

Breast cancer is one of the most common cancers in women [1]. About more than 70 % of breast cancers are ER positive [2]. The prognosis of ER positive breast cancer has been improved by endocrine therapy. Tamoxifen is the most common and effective therapy for patients with ER positive breast cancer. Alone or after chemotherapy, tamoxifen remarkably reduces disease progression and is correlated with more favorable influence on survival in patients. Despite the truth that tamoxifen allows a significant reduction of breast cancer mortality, many patients fail to respond to the initial therapy (de novo resistance) or develop resistance after long term treatment (acquired resistance) [3]. The tamoxifen resistance is likely to reside in the expression (de novo or acquired) of specific molecules involved in different signaling pathways, which eventually could be used as predictive biomarkers of resistance. Therefore, the identification of biomarkers becomes increasingly relevant [4, 5].

Tumor microenvironment is fundamental to breast cancer development. The microenvironment of breast cancer contains tumor-associated macrophages (TAMs), cancer-associated fibroblasts endothelial cells, extra cellular matrix, tumor vasculature, and multiple non-malignant cell populations [6]. Most of them act as abettors or double-swords in the formation of breast cancer. The TAMs represent a

predominant component of the stroma in the tumor [7]. First appropriately activated macrophages, which are immunocompetent cells, can suppress tumor cells [8, 9]. However, they can also produce a great diversity of cytokines which may promote tumor progression [8, 9]. Lipopolysaccharide and Interferon-γ mediate M1 macrophages, while interleukin-4 (IL-4), IL-10, IL-13 mediate M2 macrophages [10]. TAMs are characterized mainly by M2 macrophages [11]. CD163, a scavenger receptor, is regarded as a highly specific monocyte/macrophage marker for M2 macrophages [11]. TAMs conduct a very important role in the promotion of the growth of blood vessels and are related with the worse prognosis [12, 13]. In breast cancer, some reports have demonstrated that macrophage infiltration was associated with EGFR expression. EGFR is known to be involved in tamoxifen resistance [4]. However, the precise role of TAMs in tamoxifen resistance remains unclear. In order to identify the relationship between TAMs and tamoxifen resistance, we examined the expression of epidermal growth factor receptor (EGFR) and CD163+ macrophages in 100 breast cancer tissues by immunohistochemical analysis.

## Materials and Methods

### Collection of Tissue Samples

The tumor tissue samples from patients with postmenopausal, ER-positive, Her-2-negative, infiltrating ductal carcinoma were enrolled in the study. Patients had undergone surgery in the department of Medical Oncology, at the Tumor Hospital of Harbin Medical University, from January 2001 throughout February 2003. They were confirmed to have no distant metastasis and also received no endocrine therapy and neoadjuvant chemotherapy preoperatively. Depending on the grade and stage of the breast cancer, patients were selectively given chemotherapy and radiation therapy. They were subsequently planted to be treated with endocrine therapy (using tamoxifen of 20 mg per day) for 5 years. The date of the surgery was used to represent the beginning of the follow-up period and follow ups were terminated in February 2013. Patients who developed recurrence or metastasis were identified during the regular follow-up visits by using adequate diagnostic imaging modalities and tumor-marker measurements. We selected the patients who had recurrence or metastasis after chemotherapy during the tamoxifen therapy and defined them as tamoxifen resistance(TR) group. The other patients, for control, who did not suffer recurrence or metastasis after chemotherapy during the follow-up period, were called the tamoxifen sensitive (TS) group.

### Immunohistochemical Analysis

Immunohistochemical analysis was performed as described previously [13]. In brief, slides were incubated with anti-CD163 antibody (10D6 dilution 1:250; Novocastra, Newcastle, UK) and EGFR (Ab-1070) antibody (B7060, dilution 1:100, Assay Biothech, Los Angeles, USA). Negative control was stained with phosphate-buffered saline (PBS) plus 1 % bovine serum albumin (BSA) instead of the primary antibody. Results of the analyses were evaluated by two pathologists who had no access to the clinical data. Yellow granules indicated CD163 positive. The CD163 staining was scored as the infiltration density of CD163+ macrophages ranging from 0 (absent) up to 3 (dense). Greater than or Equal to the score of 1 was regarded as TAMs positive. Yellow-brown granules were the sign of EGFR positive result. Staining of the tumor cell membranes or cytoplasm was regarded as EGFR positive [14]. Immunoreactivity was scored by the staining intensity (negative, weak, moderate, or strong staining) and the percentage of positive tumor cells per core (≤25 %; >25–50 %; >50–75 %; and >75 %). Tissues were considered positive for EGFR expression with≥moderate staining intensity in >25 % of the cells examined.

### Statistical Analysis

The data was evaluated by the Chi-square test. Assessments of the correlation were performed using Spearman rank order correlation. The time for recurrence-free survival was calculated as the time between diagnosis and local recurrence of breast cancer, distant metastasis or death from breast cancer. The survival curves were analyzed using the Kaplan-Meier method and comparison between curves was evaluated using the log-rank test.

All statistical analyses were carried out using the IBM SPSS statistics version 20.0 (SPSS Inc). If the *P*-value was <0.05, statistical significance was determined.

## Result

There were totally 100 postmenopausal, ER-positive and Her-2-negative breast tumors included in this study. The demographics of the patients and histological details of their breast cancers were described in Table 1. We found that the body mass index (BMI) was related to tamoxifen resistance ($P<0.05$). The patients who had a body-mass index more than 28 were more likely to develop to tamoxifen resistance.

CD163+ macrophages were mainly distributed along the invasive margin of the tumor, tumor stroma (Fig. 1). The staining of the breast cancer cell cytoplasm or membranes was considered to be the EGFR expression (Fig. 2).

**Table 1** Clinical feature of the two groups (TR group and TS group)

| Demographics and histology | TR group | TS group | *P* value |
|---|---|---|---|
| No of cases, n (%) | 48 | 52 | |
| Age(years)(mean/median/range) | 52/57/48-76 | 51/58/49-75 | |
| BMI | | | |
| <28 | 32 | 44 | |
| ≥28 | 16 | 8 | **0.031** |
| Grade | | | |
| 1 | 1(2) | 3(6) | |
| 2 | 43(90) | 47(90) | |
| 3 | 4(8) | 2(4) | 0.301 |
| Nodal status | | | |
| Positive | 33(73) | 27(52) | |
| Negative | 15(27) | 25(48) | 0.065 |
| Tumor size | | | |
| <2 cm | 6(12.5) | 9(17.3) | |
| 2–5 cm | 37(77.1) | 41(78.8) | |
| >5 cm | 5(10.4) | 2(3.9) | 0.186 |
| Progesterone receptor | | | |
| Positive | 39(81.3) | 44(84.6) | |
| Negative | 9(18.7) | 8(15.4) | 0.427 |
| Chemotherapy | | | |
| Anthracyclines | 39(81.3) | 50(96.1) | |
| Taxanes | 30(62.5) | 28(53.8) | 0.221 |

*TR group* tamoxifen resistance group; *TS group* tamoxifen sensitive group; *BMI* body mass index

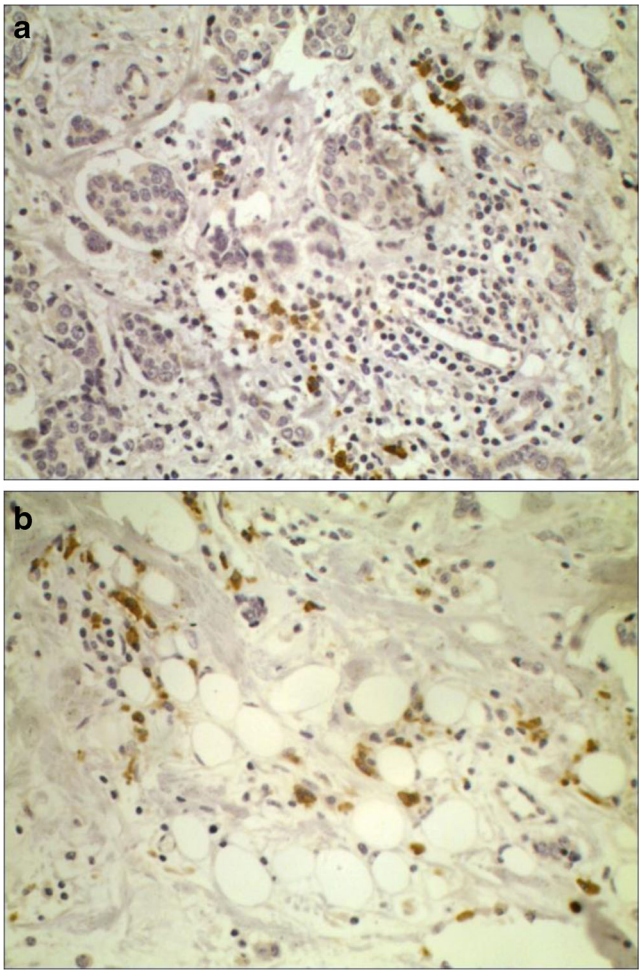


**Fig. 1** CD163+ macrophages were present in breast cancer (×400)(**a**,**b**)

EGFR was found to be positive in 21(43.8 %) and CD163+ macrophages were observed in 26 (54.2 %) of 48 cases in TR group. Positive staining for EGFR and CD163+ macrophages were observed in 6 (11.5 %) and in 12 (23.1 %) of 52 tumors in TS group. The expression of EGFR and CD163+ macrophages in TR group was higher than that of TS group ($P$=0.000279 and $P$=0.001 respectively) (Tables 2 and 3). By using the Spearman method, significant positive correlations were found between TAMs and EGFR ($r$=0.567,$P$<0.01). TAMs were positively correlated with tumor size, lymph node metastasis and BMI in enrolled patients (Table 4).

Only patients in the TR group were included into the Kaplan-Meier test ($n$=48). The patients with higher density of CD163+ macrophages infiltration suffered from shorter time to develop recurrence or metastasis ($p$<0.05) (Fig. 3).

## Discussion

The mechanisms that contribute to endocrine resistance include loss or modification of ER expression, regulation of signal transduction pathways, alerted expression of specific microRNAs, balance of co-regulatory proteins, and genetic polymorphisms involved in tamoxifen metabolic activity [15].

ER is associated with cell propagation and survival by two different mechanisms: genomic and non-genomic signaling pathway. Both ways interact with each other in crosstalk. Whereas nuclear ER causes the expression of transforming growth factor(TGF)-α and amphiregulin(AR), both TGF-α and AR are able to bind and stimulate EGFR or EGFR/Her2 and consequently activate MAPK and AKT [16–18]. On the other hand, membrane ER can bind to caveolin 1 and activate specific G proteins, which activates matrix metalloproteinases that separate transmembrane precursors of heparin binding-EGF, an EGFR ligand [19]. To date, most studies have revealed that the EGFR pathway is the principal growth factor receptor pathway in tamoxifen resistance.

According to our data, the tissues were the overexpression of EGFR in the TR group, which had been a consistent feature in numerous studies [20]. One study even indicated that EGFR expression could be applied to predict tamoxifen resistance [21].

Macrophages incessantly infiltrate into the tumor microenvironment where tumor cells and other infiltrates release factors that lead macrophages to TAMs which are thought both

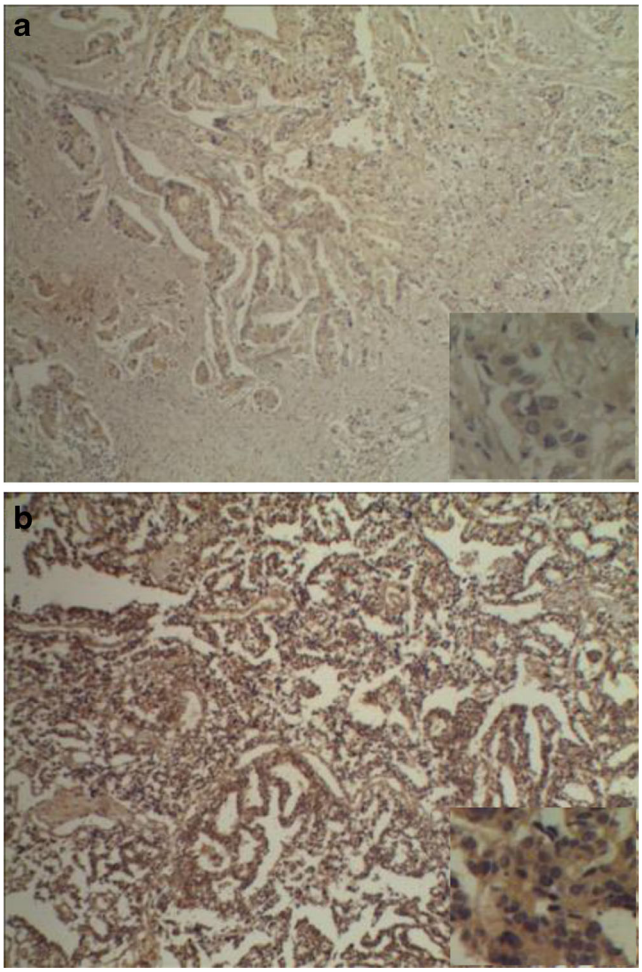


**Fig. 2** Different patterns of EGFR immunohistochemical staining (×100,×400). **a** Weak EGFR expression in breast cancer. **b** Strong EGFR expression in breast cancer

M1 and M2 macrophages. M1 macrophages are pro-inflammatory and characterized by high expression of pro-inflammatory factors [22]. Contrarily, M2 macrophages are immunosuppressive. They can produce high levels of antiinflammatory cytokines [22]. TAMs are macrophages of the M2 phenotype which, in contrast to circulating macrophages of M1 phenotype, possess poor antigen-presenting ability. TAMs produce inflammatory cytokines and angiogenic factors, which are able to promote tumor progression and metastasis. In our study, we found that TAMs were positively correlated with tumor size and lymph mode

**Table 2** Difference in CD163+ macrophages between TR group and TS group

| | CD163(+) | CD163(-) | n | Positive rate(%) | $\chi 2$ | *p* |
|---|---|---|---|---|---|---|
| TR group | 26 | 22 | 48 | 54.2 | 10.240 | 0.001 |
| TS group | 12 | 40 | 52 | 23.1 | | |

**Table 3** Difference in EGFR expression between TR group and TS group

| | EGFR (+) | EGFR (-) | n | Positive rate(%) | $\chi 2$ | *p* |
|---|---|---|---|---|---|---|
| TR group | 21 | 27 | 48 | 43.8 | 13.140 | 0.000279 |
| TS group | 6 | 46 | 52 | 11.5 | | |

metastasis. The result that we got was in agreement with previous studies [23, 24].

In the study, we also found that BMI was related to tamoxifen resistance and TAMs. Obesity is defined as a BMI of 28 or more in China. In postmenopausal women, increased BMI has been associated with adipocyte hypertrophy in the breast [25]. Adipocytes can release a variety of adipokines and proinflammatory cytokines, which are likely to contribute to the recruitment and activation of immune cells including macrophages [26]. Adipocyte death leads to myeloid cell recruitment in a characteristic pattern whereby macrophages form a crown surrounding the dead adipocyte. This formation is histologically apparent as crown-like structures (CLS), which have been observed in subcutaneous and visceral fat in association with the metabolic syndrome [27]. The presence of CLS in women is associated with activation of NFκB and increased levels of TNFα, IL-1β, IL-6, and PGE2 which are known to upregulate aromatase expression via specific promoters that give rise to unique mRNA species found in breast tissue [28, 29]. Increased levels and activity of aromatase lead to enhanced estrogen biosynthesis and upregulation of PR, an ER target gene. Additionally, increased circulating levels of proinflammatory mediators in obese patients with breast cancer correlate with poor prognosis [30, 31]. TAMs play a key role in the occurrence and development of the postmenopausal breast cancer.

We also correlated EGFR expression with TAMs. The result showed that EGFR expression was associated with TAMs. It was the first study to explore the relationship between macrophage infiltration and Tamoxifen resistance. There were more infiltrating CD163+ macrophages in TR group. The density of CD163+ macrophages was significantly associated with recurrence-free survival rate. Our findings were consistent with Russell D.Leek et al. who also found that increased EGFR was related to more TAMs [32]. Epidermal growth factor (EGF)-immunodepletion weakened macrophage-mediated cancer cell invasion and motility, implying that EGF was the pro-invasive and pro-motile factor released by macrophages. Yang Jian et al., identified a novel EGFR/signal transducers and activators of transcription 3 (Stat3)/Sox-2 paracrine signaling pathway between macrophages and mouse breast cancer cells [33]. Philip Vlaicu et al., had analyzed transcript and secreted protein levels of Stat3 activators and of EGFR family ligands. They reported

**Table 4** Correlation between clinicopathological feature and CD163+ macrophages

| Clinicopathologic features | Number of patients(%) (Total=100) | Number of positive CD163 staining (%) | Number of negative CD163 staining(%) | *P* value |
|---|---|---|---|---|
| Grade | | | | |
| 1 | 4 | 0 | 4(100) | |
| 2 | 90 | 34(37.8) | 56(62.2) | |
| 3 | 6 | 4(66.7) | 2(33.3) | 0.142 |
| Nodal status | | | | |
| 1(negative) | 40 | 9(22.5) | 31(77.5) | |
| 2(1-3 LN) | 38 | 17(44.7) | 21(55.3) | ***r*=0.261** |
| 3(>3 LN) | 22 | 12(54.5) | 10(45.5) | **0.008** |
| Tumor size | | | | |
| <2 cm | 15 | 2(13.3) | 13(86.7) | |
| 2–5 cm | 78 | 32(41.0) | 46(59.0) | ***r*=0.213** |
| >5 cm | 7 | 4(57.1) | 3(42.9) | **0.028** |
| **BMI** | | | | |
| **<28** | **76** | **23(30.3)** | **53(69.7)** | ***r*=0.284** |
| ≥28 | 24 | 15(62.5) | 9(37.5) | **0.005** |
| Progesterone receptor | | | | |
| Positive | 83 | 29(34.9) | 54(65.1) | |
| Negative | 17 | 9(52.9) | 8(47.1) | 0.132 |

Significant *P* values are in bold

*r* Spearman rank order correlation

*LN* lymph node

*BMI* body mass index

that TAMs co-expressed oncostatin-M and heparin-binding EGF-like growth factor (HB-EGF) in primary mammary carcinoma samples. In these patients, HB-EGF plasma protein levels strongly correlated with TAMs infiltration levels, indicating that TAM-derived HB-EGF supported carcinoma progression [34]. Condeelis et al., demonstrated that colony-stimulating factor 1, released by tumor cells, and EGF, released by the TAMs, acted on the reciprocal cell types to

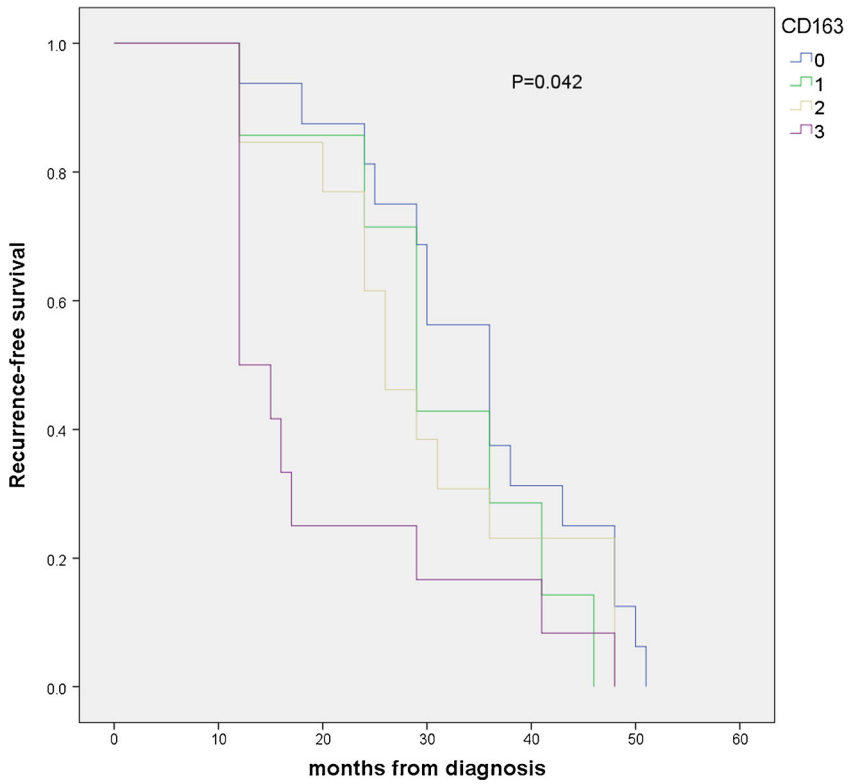


**Fig. 3** Kaplan-Meier curves representing the cumulative recurrence-free survival in tamoxifen resistance patients that were scored for the infiltration density of CD163+ macrophages

activate tumor cell migration [35]. TAMs were a main source of EGF in breast cancer. EGFR and TAMs were highly expressed in TR group. The tamoxifen resistance related to EGFR expression. In the future, TAMs may be a potential marker to predict tamoxifen resistance.

In conclusion, we found that the number of TAMs and the expression of EGFR increased in tamoxifen resistance group. The positive correlation between TAMs and EGFR expression could be a characteristic of tamoxifen resistant tumors. Obesity was related to tamoxifen resistance and TAMs. TAMs can be regarded as therapeutic targets. Further studies are needed to investigate the potential mechanism between TAMs and tamoxifen resistance in a large cohort.

**Acknowledgments** This experiment was finished in the oncology key lab of Heilongjiang province institution of higher Education. This study was supported by the National Natural Science Foundation of China (Grant No. 81372839).

**Conflict of Interest** The authors have no conflict of interest.

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