High expression of CD47 in triple negative breast cancer is associated with epithelial-mesenchymal transition and poor prognosis

JINGPING YUAN1*, XUEHUI SHI2*, CHUANG CHEN3, HUIHUA HE1, LIN LIU1, JUAN WU1 and HONGLIN YAN1

1Department of Pathology, Renmin Hospital of Wuhan University, Wuhan, Hubei 430060; 2College of Chemistry and Molecular Sciences, Wuhan University, Wuhan, Hubei 430072; 3Department of Breast and Thyroid Surgery, Renmin Hospital of Wuhan University, Wuhan, Hubei 430060, P.R. China

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Abstract. CD47 functions as a dominant anti-engulfment signal on tumour cells and is overexpressed in various malignant tumours. However, the expression and functional significance of CD47 in triple-negative breast cancer (TNBC) is not completely understood. In the present study, CD47 was demonstrated to be overexpressed in TNBC solid tumours. Moreover, increased CD47 expression was significantly associated with an advanced tumour-node-metastasis stage, lymph node involvement and recurrence. Moreover, CD47 was an unfavourable and independent prognostic factor for 5-year disease-free survival in patients with TNBC. Thus, CD47 may be a potential prognostic biomarker and therapeutic target for TNBC.

Introduction

At present, breast cancer is the most common type of cancer in women and one of the leading causes of cancer-associated mortality in women worldwide, despite improvements in diagnostic techniques and treatment modalities (1). The progression and metastasis of breast cancer are the main causes of mortality. Based on cellular markers reflecting the available targeted therapies, breast cancer is characterised as oestrogen receptor (ER)- or progesterone receptor (PR)-positive, human epidermal growth factor receptor 2 (HER2)-positive and triple-negative breast cancer (TNBC) (2). Targeted therapy can be implemented for ER/PR-positive and HER2-positive breast cancer; however, since TNBC is defined by the absence of the ER, PR and HER2 genes, there is no standard treatment for TNBC at present (3). The lack of targeted therapies and the poor prognosis in patients with TNBC has prompted major research efforts to identify new molecular targets of TNBC.

Studies have shown that functional changes in the immune system play an important role in the occurrence and development of carcinoma, where immunotherapy was a breakthrough point in cancer treatment (4). CD47, also known as integrin-associated protein, is a transmembrane protein that is highly expressed on the surface of various cancer cells. It interacts with thrombospondin-1, signal-regulatory protein-α (SIRP-α) and other proteins to regulate various cellular functions, such as T cell activation and cell migration (5). Emerging evidence has indicated the function of CD47 as a dominant anti-engulfment signal on tumour cells, by binding with SIRP-α on phagocytic immune cells to prevent engulfment (6-8). Given that the expression of CD47 was inhibited in certain malignant tumours, the blockade of the CD47 anti-engulfment signal increased the phagocytosis of tumour cells in solid tumours and haematological malignancies (6). Therefore, CD47 is a protective therapeutic target on solid tumour cells.

Although breast cancer was not considered an immunogenic malignant tumour, certain studies have revealed an association between the intratumoural immune response and tumour progression (9-11). Since TNBC and HER2-positive breast cancer are highly proliferative, increased genomic instability and mutational burden may result in the exposure of a large number of tumour antigens and promote the anti-tumour immune response (12). Thus, TNBC and HER2-positive breast cancer were considered to be more immunogenic than other types of breast cancer (12). Although studies have shown the abnormal expression of CD47 in breast cancer cell lines, breast cancer stem cells, bone marrow, peripheral blood and the circulating tumour cells of breast cancer (13-17), most of these studies focused on the association between CD47 and breast cancer at the cytological level. There is little direct
Evidence of the expression of CD47 in breast cancer solid tumours, particularly in TNBC solid tumours, and its association with metastasis, survival rate and prognosis have rarely been reported.

Epithelial-mesenchymal transition (EMT) is a favourable explanation for the distant metastasis of breast cancer (18). The essential characteristics of EMT include the destruction of tight junctions and the loss of cell-to-cell contact, which leads to the loss of epithelial characteristics (such as the loss of the epithelial cell adhesion protein E-cadherin) and the acquisition of a mesenchymal morphology (such as the gain of the mesenchyme-associated molecule N-cadherin) (18). EMT can be induced by a variety of stimulants, such as cytokines and growth factor signalling molecules, and transforming growth factor-β (TGF-β) is strongly involved in the induction of EMT in epithelial cells (including breast cancer epithelial cells) (19). However, the association between the expression of CD47 and EMT in breast cancer was rarely reported. In the present study, the expression of CD47 and its prognostic significance in TNBC solid tumours was evaluated, as well as the association between CD47 and EMT, in order to explore novel mechanisms for the development of TNBC therapies.

Materials and methods

Tissues. Formalin-fixed, paraffin-embedded tissue samples from 57 patients with TNBC, who underwent surgery for primary breast cancer, were randomly selected from the medical records of the Department of Pathology, Renmin Hospital of Wuhan University between August 2009-December 2010. The inclusion criteria were as follows: i) Received no treatment prior to surgery; and ii) female. Patient ages were in the range of 31-69 years (mean, 50.2 years; median, 42 years). Major clinicopathological parameters, including age, menopausal status, tumour-node-metastasis (TNM) stage, histological grade, lymph node metastasis and recurrence, are summarized in Table II. TNM stage was grouped according to the American Joint Committee on Cancer 7th Edition Cancer Staging Manual 2010 (20). The histological grade was classified in accordance with the 4th edition of the WHO histological grading (21). Each patient was followed up for 5 years after surgery and the survival times were recorded. A total of 40 cases of benign breast lesions were also randomly selected from Department of Pathology, Renmin Hospital of Wuhan University between August 2009 and December 2010 as controls. The ages were in the range of 19-64 years, with a mean age of 35 years. Written informed consent was obtained from the patients, and the study was approved by the Ethics Committee of Renmin Hospital of Wuhan University.

Immunohistochemistry (IHC). IHC was performed to detect CD47, E-cadherin, N-cadherin and TGF-β. Formalin-fixed, paraffin-embedded tissue samples were cut into 4 μm sections and then deparaffinised with xylene twice for 10 min and rehydrated with 100% ethanol twice for 5 min, 95% ethanol twice for 2 min and 85% ethanol for 2 min at room temperature. Following that, the sections were blocked with 3% H2O2 for 10 min at room temperature to block endogenous peroxidase activity and then subjected to antigen retrieval in citrate buffer (pH 6.0) at 98°C for 15 min. This was followed by incubation with primary antibodies: Anti-CD47 (1:100; cat. no. sc-12730; Santa Cruz Biotechnology, Inc.); anti-E-cadherin (1:100; cat. no. ab1416; Abcam); anti-N-cadherin (1:500; cat. no. ab18203; Abcam); or anti-TGF-β (1:100; sc-130348; Santa Cruz Biotechnology, Inc.).

Staining evaluation. Immunohistochemical staining of all sections were evaluated by two independent pathologists (JY and HH). The evaluating pathologists were blinded to the clinical data. In the present study, the expression of CD47 was mainly localised to the cytoplasm or cell membrane, which appeared yellow or brown. The degree of CD47 reactivity was scored by applying a semi-quantitative immunoreactivity scoring (IRS) system as described by Baccelli et al (13). The staining intensity was categorized into four grades: 0, no immunostaining; 1, weak staining; 2, moderate staining; and 3, strong staining. The percentage of positive cells was categorized into five grades: 0, 0%; 1, 1-10%; 2, 11-50%; 3, 51-80%; and 4, >80%. The staining intensity and percentage of positive cells were multiplied to obtained an IRS, in the range of 0-12 for each individual case. A case was scored as positive for CD47 with an IRS between 7 and 12 and negative with an IRS between 0-6 (13).

E-cadherin-positive cells contained yellow or dark brown granules on the cell membrane. N-cadherin-positive cells exhibited a yellow or dark brown colour in the cytoplasm or at the cellular membrane. According to the method described by Nakajima et al (22) and Liu et al (23), E-cadherin expression was considered to be normal if ≥90% of cancer cells exhibited a staining pattern similar to that in normal epithelial cells, and sections with <90% of the cancer cells stained or with a complete absence of staining were classified as a reduced pattern. Therefore, ≥90% indicated positivity and <90% indicated negativity for E-cadherin expression. Similarly, according to the method described by Liu et al (23), the proportion of positive cells ≥5% indicated positivity, whereas <5% indicated negativity for N-cadherin expression.

TGF-β was primarily located in the cytoplasm or nucleus and was indicated by a yellow or brown colour. The immunohistochemical staining of tumour cells was evaluated semi-quantitatively, as described by Chen et al (24), based on: i) Staining intensity, where 0, No staining; 1, weak staining; 2, moderate staining; and 3, strong staining; and ii) extent of the staining, where 0, ≤5%; 1, 6-25%; 2, 26-50%; 3, 51-75%; and 4, 76-100%. The final score was obtained by multiplying the scores for both the staining intensity and the extent of the staining; a final score ≤2 indicated negative staining, and >2 indicated positive staining.

Statistical analysis. The χ2 test was used to compare the CD47 expression between TNBC tissues and benign breast lesions,
analyse the association between CD47 expression and the clinicopathological parameters, and evaluate the association between CD47 expression and EMT markers. Kaplan-Meier analysis and the log-rank test were used for survival analysis. The prognostic value of CD47 was estimated by univariate and multivariate Cox proportional hazard regression analysis. All statistical analyses were performed with SPSS software version 19.0 (IBM Corp.). P<0.05 was considered to indicate a statistically significant difference. Data are presented as the mean ± standard error of the mean. The experiments were repeated in triplicate.

**Results**

**CD47 is highly expressed in TNBC tissues.** The expression of CD47 was detected in 40 benign breast lesions and 57 TNBC tissues (Fig. 1A and B). In TNBC tissues, CD47 was mainly expressed in the cytoplasm or at the cell membrane in brown granules (Fig. 1A), and positive immunohistochemical expression of CD47 was found in 44/57 (77.2%) cases. The immunoreactivity for CD47 in benign breast lesions was negative or weakly positive in the cytoplasm (Fig. 1B). The CD47-positive rate in 40 cases of benign breast lesions was 30% (12/40 cases). The CD47-positive rate was significantly higher in TNBC tissues than in benign breast lesions ($\chi^2=21.453; P<0.001$; Table I).

**Association between CD47 expression and clinicopathological parameters of TNBC.** The association between CD47 expression and clinicopathological parameters were further analysed in 57 TNBC tissues (Table II). Positive staining for CD47 was significantly associated with advanced TNM stage ($\chi^2=7.241; P=0.027$), lymph node involvement ($\chi^2=4.403; P=0.036$) and recurrence ($\chi^2=5.900; P=0.015$). The CD47-positive rate increased with increasing TNM stage (0, 71.1 and 94.4%). The CD47-positive rate in patients with lymph node metastasis or recurrence was significantly higher than in patients without lymph node metastasis or recurrence. There was no significant association between positive staining for CD47 and other clinicopathological parameters, such as patient age, menopause and histological grade (P>0.05).

**Association between CD47 and EMT markers.** Currently, EMT is a favourable explanation for the distant metastasis of epithelial cancers, including TNBC. The loss of E-cadherin and the gain of N-cadherin expression are markers of EMT. Consistent with previous reports, the expression of E-cadherin was decreased (Fig. 1C), whereas the expression of N-cadherin was increased (Fig. 1D) in TNBC tissues. The TGF-β pathway has been strongly implicated in inducing EMT in epithelial cells (17). Thus, the expression of TGF-β was also examined in TNBC tissues (Fig. 1E).

The association between the expression of CD47 and EMT markers was investigated (Table III). The expression of CD47 was associated with decreased expression of E-cadherin ($\chi^2=4.414; P=0.036$) and increased expression of N-cadherin ($\chi^2=9.216; P=0.002$). CD47 expression was associated with increased expression of TGF-β ($\chi^2=8.093; P=0.004$). These results suggested that the expression of CD47 may be involved in the process of EMT in TNBC.

**Expression of CD47 is a marker of poor prognosis in TNBC.** The prognostic value of CD47 was determined in 57 TNBC cases, following a 5-year follow-up. Kaplan-Meier analysis and the log-rank test showed a statistically significant association between a high expression level of CD47 and a low 5-year disease-free survival (5-DFS) time (P=0.034; Fig. 2), indicating that a high expression level of CD47 may serve as a novel marker of TNBC with a poor clinical outcome. To further validate the prognostic significance of this new parameter, CD47 and all clinicopathological features, including age, menopausal status, TNM stage, histological grade, lymph node metastasis and recurrence, were subjected to univariate and multivariate Cox proportional hazard regression analysis. As presented in Table IV, univariate Cox proportional hazard regression analysis confirmed that an advanced
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TNM stage (P<0.001; HR=5.964; 95% CI=2.709-13.129), the presence of lymph node metastasis (P<0.001; HR=4.596; 95% CI=2.096-10.079), recurrence (P<0.001; HR=64.448; 95% CI=9.512-436.670), and positive CD47 expression (P=0.042; HR=2.464; 95% CI=1.032-5.882) were significantly associated with a poor 5-DFS time in patients with TNBC. Multivariate statistical analysis confirmed that recurrence (P<0.001; HR=91.009; 95% CI=9.950-832.400) and positive CD47 expression (P=0.037; HR=3.432; 95% CI=1.079-10.922) were significant independent predictors of poor 5-DFS time.

Discussion

TNBC accounts for 15% of all breast cancer cases and is characterised by early relapse and metastasis (25). The ability of cancer cells to escape the innate and adaptive immune systems plays a key role in the formation of secondary (recurrent and/or metastatic) tumours (26,27). Lehmann et al (28) reported that the loss of immune infiltration in tumours was associated with mortality in patients with TNBC. One of the main mechanisms regulating the escape of cancer cells from innate immunity is the expression of CD47, which interacts with SIRP-α on macrophages to prevent phagocytosis. Shuptrine et al (29) identified 709 genes that selectively regulated adaptive anti-tumour immunity in a syngeneic TNBC model, by developing an unbiased, in vivo, genome-wide RNA interference screening platform. Of the five genes with the greatest impact identified by the screening, CD47 had the greatest impact on the immune regulatory pathway (29).

CD47 was first described as a tumour antigen in human ovarian cancer in the 1980s (30). Since then, CD47 has been found to be highly expressed in various types of human cancer,
including non-Hodgkin’s lymphoma (31), acute myeloid leukaemia (32), hepatocellular carcinoma (33,34) and bladder cancer (35), and its expression is closely associated with the differentiation, metastasis, survival and prognosis of tumours. Accordingly, CD47 is considered a biomarker of cancer, and its high expression is an indicator of poor clinical prognosis. In breast cancer, the abnormal expression of CD47 has been detected in various breast cancer-associated cells, including in breast cancer cell lines (36), breast cancer stem cells (17), bone marrow (14), peripheral blood (14) and circulating tumour cells (13,16) of breast cancer. However, most studies have evaluated the association between CD47 and breast cancer at the cytological level, and few have reported on the expression of CD47 in solid breast cancer tumours, particularly TNBC. The present study was the first, to the best of our knowledge, to reveal the overexpression of CD47 in TNBC solid tumour tissues compared with its expression in benign breast lesions.

CD47 is a novel prognostic biomarker of certain malignant tumours. In circulating colorectal cancer cells, the upregulation of the CD47 gene was associated with distant metastasis as a potential immune escape mechanism (37). In non-small cell lung cancer, increased CD47 expression was associated with clinical staging, T classification, lymph node metastasis and distant metastasis (38). Consistent with the above reports, the overexpression of CD47 in TNBC was significantly associated with TNM stage, lymph node involvement and recurrence in the present study. In addition, high CD47 expression levels have been reported to be an unfavourable independent prognostic factor for 5-DFS time (14). A previous study on patients with breast cancer indicated the low survival rate of patients with high CD47 expression in the bone marrow and circulating tumour cells than that of patients with low CD47 expression (14). In the present study, Kaplan-Meier analysis and the log-rank test demonstrated a poor 5-DFS time in patients with TNBC and high expression levels of CD47. A study by Baccelli et al (13) demonstrated the co-expression of MET-CD47 as an independent prognostic factor for the overall survival (OS) of patients with breast cancer, and that this was associated with lymph node metastasis. It was found that the OS of patients with MET-CD47 double-positive luminal breast cancer was 10.3 years lower than in those with MET-CD47 double-negative expression. Further investigation in the present study, through multivariate statistical analysis, indicated the expression of CD47 in TNBC as an independent significant predictor of poor 5-DFS time. Overall, these findings revealed the potential of CD47 as a prognostic marker in TNBC.

High expression of CD47 in various tumours indicates poor prognosis; however, the underlying mechanism of the transcriptional regulation of CD47 remains unclear. EMT is regulated by a series of EMT-induced transcription factors that promote immune escape and drug resistance (39). Noman et al (40) reported the upregulation of CD47 in various EMT-activated human breast cancer cells and in the inhibition of phagocytosis. Li et al (41) demonstrated the induction of EMT by CD47, through the modulation of E-cadherin and N-cadherin, which contribute to the invasion of high-grade serous ovarian carcinoma. This suggests a potential association between CD47 expression and EMT. Consequently, the association between CD47 expression in TNBC and several markers of EMT was evaluated in the present study. The expression of CD47 was associated with high expression of N-cadherin and TGF-β1, in contrast with decreased expression of E-cadherin. Thus, the expression of CD47 may be involved in the process of EMT in TNBC. Mechanistically, SNAI1 and ZEB1 have been reported to be major regulators of CD47 (40). The overexpression of SNAI1 or ZEB1 in human breast cancer epithelial cells results in the activation of EMT and the upregulation of CD47, by binding directly to the E-box-2 and E-box-3 motifs of the human CD47 proximal promoter (40). This indicates an EMT-dependent upregulation of CD47 in breast cancer. On the other hand, Shinohara et al (42) demonstrated the co-localization of CD47 with E-cadherin at cell-cell adhesion sites in mouse

| Protein   | CD47 protein expression | Positive | Negative | χ²  | P-valuea |
|-----------|-------------------------|----------|----------|-----|----------|
| E-cadherin| Positive, n             | 13       | 8        | 4.414 | 0.036    |
|           | Negative, n             | 31       | 5        |      |          |
|           | Positive rate, %        | 29.55    | 61.54    |      |          |
| N-cadherin| Positive, n             | 38       | 6        | 9.216 | 0.002    |
|           | Negative, n             | 6        | 7        |      |          |
|           | Positive rate, %        | 86.36    | 13.64    |      |          |
| TGF-β     | Positive, n             | 35       | 5        | 8.093 | 0.004    |
|           | Negative, n             | 9        | 8        |      |          |
|           | Positive rate, %        | 79.55    | 20.45    |      |          |

aχ² test. TGF-β, transforming growth factor-β.

Figure 2. Effect of CD47 expression on the 5-DFS times of patients with TNBC. Kaplan-Meier survival analysis and log-rank test demonstrating the association between CD47 expression and the 5-DFS times of patients with TNBC. 5-DFS, 5-year disease-free survival; TNBC, triple-negative breast cancer.
breast cancer-derived epithelial cells. Since E-cadherin was known to be involved in the actin cytoskeleton of epithelial cells, CD47 was found to reorganize the actin cytoskeleton to participate in the regulation of cell-cell adhesion and cell migration (42). Based on the findings of the present study and those from the literature, the involvement of CD47 in the process of EMT in TNBC is speculated. Whether CD47 promotes EMT in epithelial cells, or whether EMT-associated transcription factors or proteins regulate the expression of CD47, is yet to be determined.

At present, there are few studies on the expression of CD47 in TNBC solid tumours and its association with EMT. In the present study, the overexpression of CD47 was demonstrated in TNBC, which was associated with advanced TNM stage, lymph node involvement, recurrence and a reduced 5-DFS time. In addition, the expression of CD47 was associated with several EMT markers in TNBC. Overall, these findings suggest the potential of CD47 as a prognostic marker and therapeutic target for TNBC.

However, some limitations of the present study should be acknowledged. Firstly, the sample size is small. The sample number will be increased in order to explore the role and mechanism of CD47 in the progression of breast cancer in the future. Secondly, the association between CD47 expression and prognosis was evaluated using 5-DFS data, rather than OS. Since DFS is not directly associated with OS, the effect of CD47 expression on OS remains a topic for future studies. Therefore, the results of the study should be interpreted with caution, and further validation is warranted with a larger sample size and longer clinical follow-up time.

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Availability of data and materials
The datasets used and/or analysed during the present study are available from the corresponding author on reasonable request.

Authors’ contributions
CC, LL and JW collected the samples and performed the statistical analysis; JY and HH evaluated the immunohistochemical staining of all sections; JY, XS and HY designed the study and wrote the manuscript; HY revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The present study was approved by the Ethics Committee of Renmin Hospital of Wuhan University (Wuhan, China) (approval no. WDRY2019-K010). Patients provided written informed consent for the use of their tissues in the present study.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Table IV. Univariate and multivariate Cox proportional hazard regression analysis of 5-year disease-free survival times of patients with triple-negative breast cancer.

| Variable                             | Univariate HR | Lower CI 95% | Upper CI 95% | P-value | Multivariate HR | Lower CI 95% | Upper CI 95% | P-value |
|--------------------------------------|---------------|--------------|--------------|---------|----------------|--------------|--------------|---------|
| Age (≤50 vs. >50)                    | 1.113         | 0.561        | 2.207        | 0.760   | 0.372          | 0.098        | 1.414        | 0.147   |
| Menopause (after vs. before)         | 0.698         | 0.355        | 1.373        | 0.298   | 0.386          | 0.111        | 1.340        | 0.134   |
| TNM stage (I and II vs. III)         | 5.964         | 2.709        | 13.129       | <0.001  | 1.361          | 0.524        | 3.531        | 0.527   |
| Histological grade (grade 1 vs. 2)   | 1.551         | 0.790        | 3.048        | 0.203   | 1.594          | 0.760        | 3.340        | 0.217   |
| Lymph node metastasis (no vs. yes)   | 4.596         | 2.096        | 10.079       | <0.001  | 2.255          | 0.814        | 6.243        | 0.118   |
| Recurrence (no vs. yes)              | 64.448        | 9.512        | 436.670      | <0.001  | 91.009         | 9.950        | 832.400      | <0.001  |
| CD47 (negative vs. positive)         | 2.464         | 1.032        | 5.882        | 0.042   | 3.432          | 1.079        | 10.922       | 0.037   |

CI, confidence interval; HR, hazard ratio; TNM, Tumour-Node-Metastasis.
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