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ORIGINAL RESEARCH

Prognostic value of survivin and EGFR protein expression in triple-negative breast cancer (TNBC) patients

Minghui Zhang · Xiaosan Zhang · Shu Zhao · Yan Wang · Wenyu Di · Gangling Zhao · Maopeng Yang · Qingyuan Zhang

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Abstract Triple-negative breast cancer (TNBC) is a particular type of breast cancer which is characterized by its biological aggressiveness, worse prognosis, and lack of prognostic markers or therapeutic targets in contrast with hormonal receptor-positive and human epidermal growth factor receptor 2-positive (HER2+) breast cancers. We aimed to evaluate survivin and epidermal growth factor receptor (EGFR) expression and their prognostic value and determine their relationships with the clinicopathological parameters of TNBC. A total of 136 patients who had undergone a resection of primary TNBC were enrolled at the Third Affiliated Hospital of Harbin Medical University from March 2003 to September 2005. Expression of ER, PR, HER2, EGFR, and survivin was assessed by immunohistochemistry. The association of TNBC and other clinicopathological variables and the prognostic value of survivin and EGFR expression were evaluated. Survivin was expressed in 62 (45.6 %) cases and EGFR was expressed in 82 (60.3 %) cases. Survivin expression was associated with menopausal status ($P=0.011$), tumor size ($P=0.037$), and lymph node status ($P=0.001$). EGFR expression was associated with menopausal status ($P=0.029$), lymph node status ($P=0.004$), P53 expression ($P=0.001$), Ki-67 expression ($P=0.028$), and lymphatic vascular invasion ($P=0.037$). A multivariate analysis demonstrated that tumor size (hazard ratio (HR) 1.587, 95 % confidence interval (CI) 1.081–2.330, $P=0.018$ for disease-free survival (DFS); HR 1.606, 95%CI 1.096–2.354, $P=0.015$ for overall survival (OS)), lymph node status (HR 2.873, 95%CI 1.544–5.344, $P=0.001$ for DFS; HR 2.915, 95%CI 1.553–5.471, $P=0.001$ for OS), tumor grade (HR 1.914, 95%CI 1.218–3.007, $P=0.005$ for DFS; HR 1.983, 95%CI 1.228–3.203, $P=0.005$ for OS), EGFR (HR 3.008, 95%CI 1.331–6.792, $P=0.008$ for DFS; HR 3.151, 95%CI 1.374–7.226, $P=0.007$ for OS), and survivin (HR 1.573, 95%CI 1.087–2.277, $P=0.016$ for DFS; HR 1.607, 95%CI 1.088–2.374, $P=0.017$ for OS) were of prognostic significance for disease-free and overall survival. We draw a conclusion from the present study that survivin and EGFR expression are useful prognostic markers of TNBC and might be useful for molecular targeting therapy of TNBC treatment.

Keywords Triple-negative breast cancer · EGFR · Survivin · Prognosis

Introduction

Breast cancer is a heterogeneous disease with different morphologies, molecular profiles, clinical behaviors, and responses to therapy. Triple-negative breast cancer (TNBC) is a particular type of breast cancer defined by a lack of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2)/neu amplification and comprises 12–20 % of all breast cancers [1, 2]. Most TNBCs have a basal-like molecular phenotype by gene expression profiling [3, 4]. It is characterized by its biological aggressiveness, worse prognosis, and lack of a therapeutic target in contrast with hormonal receptor-positive and HER2+ breast

M.H. Zhang, X.S. Zhang, and S. Zhao contributed equally to the work and should be considered as co-first authors.

M. Zhang · X. Zhang · S. Zhao · Y. Wang · M. Yang · Q. Zhang (✉)
Department of Medical Oncology, The Third Affiliated Hospital of Harbin Medical University, Road 150 of Nangang District, Harbin, Heilongjiang Province 150081, China
e-mail: zhma19650210@126.com

W. Di
Department of Pathology, The First Affiliated Hospital of XinXiang Medical College, WeiHui 454100, China

G. Zhao
Department of Osteology, The 371 Hospital of the Chinese People's Liberation Army, XinXiang 453000, China

cancers. The characterization of TNBC is important in evaluating patients' outcomes and developing a molecular-based medicine treatment strategy.

Extensive investigation continues to search for new markers that may improve breast cancer prognosis and therapeutic approaches. Over the past few years, several reports have suggested that survivin and epidermal growth factor receptor (EGFR) are potential prognostic factors [5–8]. Survivin and EGFR have been implicated in multiple biological processes, including angiogenesis and apoptosis that are hallmarks of cancer [9, 10].

EGFR is a receptor on cell surface and its expression has been shown to be correlated with tumor proliferation, invasion, and angiogenesis [9]. EGFR expression in breast cancer has been investigated in a variety of studies whose results suggest that a relation to aggressive tumor behavior remains ambiguous [5, 6].

Survivin is a 16.5-kDa intracellular protein that belongs to the inhibitor of apoptosis (IAP) gene family [11]. It is not detectable in most differentiated normal adult tissues but is expressed in most human cancer tissues. Its expression in cancer has been shown to be correlated with poor prognosis, cancer progression, and drug resistance [12]. Survivin overexpression has been demonstrated in a variety of histological types of tumor tissues [13–18]. The relationship of survivin expression and prognosis of breast cancer is controversial as various studies have reported it to be either irrelevant [7] or associated with poor [8, 19] or good prognosis [20].

TNBC is with poor prognosis and lacks prognostic indicators. Although a correlation between survivin and EGFR expression status has been demonstrated among all types of breast cancer, little is known about the significance of survivin and EGFR expression levels and their prognostic value in TNBC. In the present study, we aimed to evaluate survivin and EGFR expression and their prognostic value and determine their relationships with the clinicopathological parameters of TNBC.

Materials and methods

Patients

The study design followed the main criteria defined by REMARK [21]. A total of 136 patients who had undergone a resection of primary TNBC were enrolled at the Third Affiliated Hospital of Harbin Medical University from March 2003 to September 2005. All samples were examined by immunohistochemistry. All patients received radical mastectomy, modified radical mastectomy, or dissection as primary treatment. None of the patients received radiotherapy or chemotherapy before the biopsy/mastectomy procedure. All of the patients who had undergone conservative breast surgery received postoperative radiotherapy on the residual breast. Adjuvant systemic chemotherapy and/or adjuvant radiotherapy was administered as clinically indicated in accordance with standard practices during this time interval. This study was approved by the Ethics Committee of the Third Affiliated Hospital of Harbin Medical University.

Immunohistochemistry

Sections were deparaffinized by passing through xylene and rehydrated by a graded series of ethanol, followed by microwave treatment for antigen retrieval for 20 min in pepsin at room temperature (pepsin solution Digest-All3, Zymed). After a brief rinse with Tris buffer (BUF1), they were incubated with 31G7 (Zytomed). After another brief rinse with Tris buffer (BUF1), the tissue was incubated with the biotinylated secondary antibody for another 25 min. After a brief rinse with Tris buffer (BUF2), the endogenous peroxidase was inactivated with peroxidase-blocking solution, three times for 2 1/2 min each. After rinsing, the tissues were incubated for another 25 min with streptavidin conjugated with horseradish peroxidase. Visualization was performed with 3-amino-9-ethylcarbazol (AEC, Dako Cytomation, Carpinteria, CA, USA) three times for 5 min, and sections were counterstained with hematoxylin. Tumor-adjacent tissue was used as a negative control. According to Guler et al. [22], the slides were classified into scores 0 to 3.Score 0 was considered when less than 10 % of all tumor cells were stained. Score 1 was considered in cases of faint or barely perceptible membrane staining in more than 10 % of all tumor cells, and in cases of heterogenous staining, weak partial and/or complete membrane staining was shown in more than 50 % of stained cells. Score 2 was considered when moderate membrane staining was shown in more than 10 % of tumor cells, and in cases of heterogenous staining, moderate partial and/or complete membrane staining is shown in more than 50 % of stained tumor cells. Score 3 was considered in cases of strong membrane staining in more than 10 % of all tumor cells, and in cases of heterogenous staining, strong partial and/or complete membrane staining is shown in more than 50 % of all tumor cells.

For survivin staining, 6-μm tumor sections cut from formalin-fixed paraffin-embedded blocks were mounted on glass slides with a silane-treated surface and deparaffinized. After antigen retrieval by heating in citrate buffer at 60 °C in an incubator overnight, the sections were treated with 3 % H_2O_2 in methanol for 20 min to abolish endogenous peroxidase activity. Then the sections were incubated with 2 g/ml of rabbit anti-survivin polyclonal antibody (Novus Biologicals, Littleton, CO, USA) at 48 °C overnight. Biotinylated goat anti-rabbit immunoglobulin (Dako) and streptavidin–horseradish peroxidase conjugate (Amersham Biosciences) were applied at room temperature. The sections were visualized

using AEC (3-amino-9-ethylcarbazole) peroxidase substrate solution and hematoxylin counterstaining. Negative control slides without primary antibody were included for each tumor section. The level of survivin expression was scored as follows based on the criteria suggested by Lo Muzio et al. [23]: positive 50 % and negative 50 % of cancer cells were stained in the cytoplasm. Slides were considered nuclear positive when more than 5 % of all tumor cell nuclei were stained regardless of the cytoplasm staining levels.

Statistical analyses

Disease-free survival was defined as from the date of the primary surgery to the first local recurrence or distant metastasis. The overall survival was the time from the date of the primary surgery to the time of breast cancer-related death. Statistical analysis was performed using SPSS 17.0 statistical software (SPSS Inc, Chicago, IL, USA). We examined the association between TNBC and other clinicopathological variables and the significance of different prognostic markers using a chi-square test and chi-square test for trend as appropriate. The association with survival was analyzed initially using the Kaplan–Meier plot and log-rank test and also with Cox regression analysis to adjust for other prognostic indicators. Clinicopathologic factors known to be associated with prognosis, such as age group (≤50 years versus >50 years), tumor size (>2 cm versus ≤2 cm), menopausal status (premenopausal versus postmenopausal), lymph node metastasis (positive versus negative), tumor grade (G3 versus G1–G2), P53 (positive versus negative), Ki-67 (>30 % versus 30 %), CK5/6, CK14, CK17 (positive versus negative), type of surgery (breast conserving versus mastectomy), pathological stage (III versus I and II), lymphatic vascular invasion (yes versus no), histological type (ductal versus others), family history (yes versus no), and EGFR and survivin (positive versus negative) were tested in a univariate analysis. Variables that were found to be significant in the univariate analysis were then entered in a multivariate analysis. A P value of 0.05 was considered significant. Cutoff values for different biomarkers included in this study were chosen before statistical analysis.

Results

Clinicopathological features of TNBC

In total, 136 TNBC patients were included in the present study and were analyzed for EGFR and survivin expression. Patients and tumor characteristics are summarized in Table 1. All patients were women with a mean age of 48.5 years (range, 27–75 years), and the median Karnofsky performance score (KPS) was 70 % (range, 60–100 %).

Table 1 Patients (n) and their characteristics

Parameters	Number of patients (n (%))
Age group	
≤50 years	75 (55.1)
>50 years	61 (44.9)
Tumor size (cm)	
≤2	57 (41.9)
>2	79 (58.1)
Menopausal status	
Premenopause	76 (55.9)
Postmenopause	60 (44.1)
Lymph node metastasis	
Negative	53 (39.0)
Positive	88 (61.0)
Tumor grade	
G1	4 (3)
G2	60 (44.1)
G3	72 (52.9)
P53	
Negative	90 (66.2)
Positive	46 (33.8)
Proliferative fraction (Ki67)	
≤30 %	75 (55.1)
>30 %	61 (44.9)
CK5/6	
Negative	40 (29.4)
Positive	96 (70.6)
CK14	
Negative	104 (76.5)
Positive	32 (23.5)
CK17	
Negative	93 (68.4)
Positive	43 (31.6)
Type of surgery	
Breast conserving	22 (16.2)
Mastectomy	114 (83.8)
Pathological stage	
I	19 (14)
II	54 (39.7)
III	63 (46.3)
Lymphatic vascular invasion	
Yes	55 (40.4)
No	81 (59.6)
Histological type	
Ductal	110 (80.9)
Lobular	14 (10.3)
Others	12 (8.8)
Family history	
Yes	10 (7.4)
No	126 (92.6)

Survivin and EGFR expression in TNBC

Immunohistochemical positive staining of survivin protein was observed in 62 (45.6 %) cases, and immunohistochemical positive staining of EGFR protein was observed in 82 (60.3 %) cases. The expressions of EGFR and survivin in triple-negative breast cancer are shown in Fig. 1. Correlations between survivin and EGFR expression and other clinicopathologic parameters are shown in Table 2. Survivin expression was associated with menopausal status ($P=0.011$), tumor size ($P=0.037$), and lymph node status ($P=0.001$). EGFR expression was associated with menopausal status ($P=0.029$), lymph node status ($P=0.004$), P53 expression ($P=0.001$), Ki-67 expression ($P=0.028$), and lymphatic vascular invasion ($P=0.037$). Compared with EGFR-negative patients, EGFR-positive patients showed significantly poorer outcomes with respect to disease-free survival ($P=0.005$) and overall survival ($P=0.009$) (Fig. 2). Survivin-positive patients experienced shorter disease-free survival ($P=0.012$) and poorer overall survival ($P=0.016$) than did survivin-negative patients (Fig. 3).

Multivariate analysis of prognostic factors

Using the Cox proportional hazards model, we performed a multivariate analysis to assess the independent predictive value of all significant markers for the overall survival and disease-free survival. Tumor size (hazard ratio (HR) 1.587, 95 % confidence interval (CI) 1.081–2.330, $P=0.018$ for disease-free survival (DFS); HR 1.606, 95%CI 1.096–2.354, $P=0.015$ for overall survival (OS)), lymph node status (HR 2.873, 95%CI 1.544–5.344, $P=0.001$ for DFS; HR 2.915, 95%CI 1.553–5.471, $P=0.001$ for OS), tumor grade (HR 1.914, 95%CI 1.218–3.007, $P=0.005$ for DFS; HR 1.983, 95%CI 1.228–3.203, $P=0.005$ for OS), EGFR (HR 3.008, 95%CI 1.331–6.792, $P=0.008$ for DFS; HR 3.151, 95%CI 1.374–7.226, $P=0.007$ for OS), and survivin (HR 1.573, 95%CI 1.087–2.277, $P=0.016$ for DFS; HR 1.607, 95%CI 1.088–2.374, $P=0.017$ for OS) were confirmed to be an independent predictor of PFS and OS (Table 3).

Discussion

Triple-negative breast cancer has a poor prognosis and is insensitive to most available hormonal or targeted therapeutic agents [24, 25]. Little is known about TNBC and few targeted therapies are available. The TNBC phenotype is heterogeneous from a histopathological and molecular perspective, which suggests that molecular subsets exist. Thus, the identification of molecular predictive signatures is necessary and will allow for the characterization of TNBC and the design of optimal treatment modalities. As referred in the "Introduction" section, EGFR expression in breast cancer has been investigated in a variety of studies whose results suggest that a relation to aggressive tumor behavior remains ambiguous. The relationship of survivin expression and prognosis of breast cancer is controversial as various studies have reported it to be either irrelevant or associated with poor or good prognosis. And most importantly, to the best of our knowledge, we are the first to address a systematic evaluation of these two candidate markers in TNBC. In the present study,

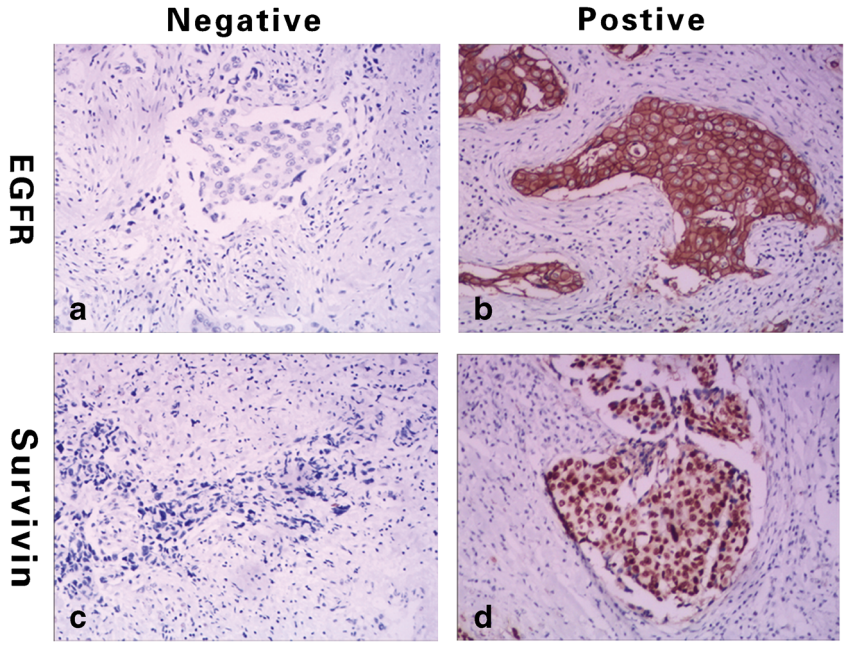

Fig. 1 The expression of EGFR and survivin in triple-negative breast cancer. A×100 magnification of immunohistochemical analysis was used. **a** EGFR-negative expression in TNBC. **b** EGFR-positive expression in TNBC. **c** Survivin-negative expression in TNBC. **d** Survivin-positive expression in TNBC

Table 2 Correlations between survivin and EGFR expression and other clinicopathologic parameters

Clinicopathologic characteristics	Survivin		*x*	*P* value	EGFR		*x*	*P* value
	(+)	(−)			(+)	(−)		
Age group			3.769	0.052			0.393	0.531
≤50 years	40	35			47	28		
>50 years	22	39			35	26		
Menopausal status			6.501	0.011			4.753	0.029
Premenopausal	42	34			52	24		
Postmenopausal	20	40			30	30		
Tumor size (cm)			10.822	0.037			0.707	0.4
≤2	20	37			32	25		
>2	42	37			50	29		
Lymph node metastasis			10.462	0.001			8.174	0.004
Negative	15	38			24	29		
Positive	47	36			58	25		
Pathological stage			4.598	0.1			4.045	0.132
I	5	14			6	13		
II	24	30			24	30		
III	33	30			52	11		
Tumor grade			1.352	0.509			3.158	0.206
G1	2	2			1	3		
G2	24	36			34	26		
G3	36	36			47	25		
P53			2.15	0.143			11.778	0.001
Negative	37	53			45	45		
Positive	25	21			37	9		
Proliferative fraction (Ki67)			2.642	0.104			4.805	0.028
≤30 %	35	40			39	36		
>30 %	37	24			43	18		
CK5/6			2.561	0.11			0.663	0.415
Negative	14	26			22	18		
Positive	48	48			60	36		
CK14			0.057	0.811			0.898	0.343
Negative	48	56			65	39		
Positive	14	18			17	15		
CK17			1.582	0.208			2.357	0.125
Negative	39	54			52	41		
Positive	23	20			30	13		
Type of surgery			3.549	0.06			2.414	0.12
Breast conserving	6	16			10	12		
Mastectomy	56	58			72	42		
Lymphatic vascular invasion			2.987	0.084			4.346	0.037
Yes	30	25			39	16		
No	32	49			43	38		
Histological type			5.47	0.065			1.26	0.533
Ductal	51	59			66	44		
Lobular	3	11			10	4		
Others	8	4			6	6		
Family history			2.593	0.107			1.751	0.186
Yes	7	3			8	2		
No	55	71			74	52		

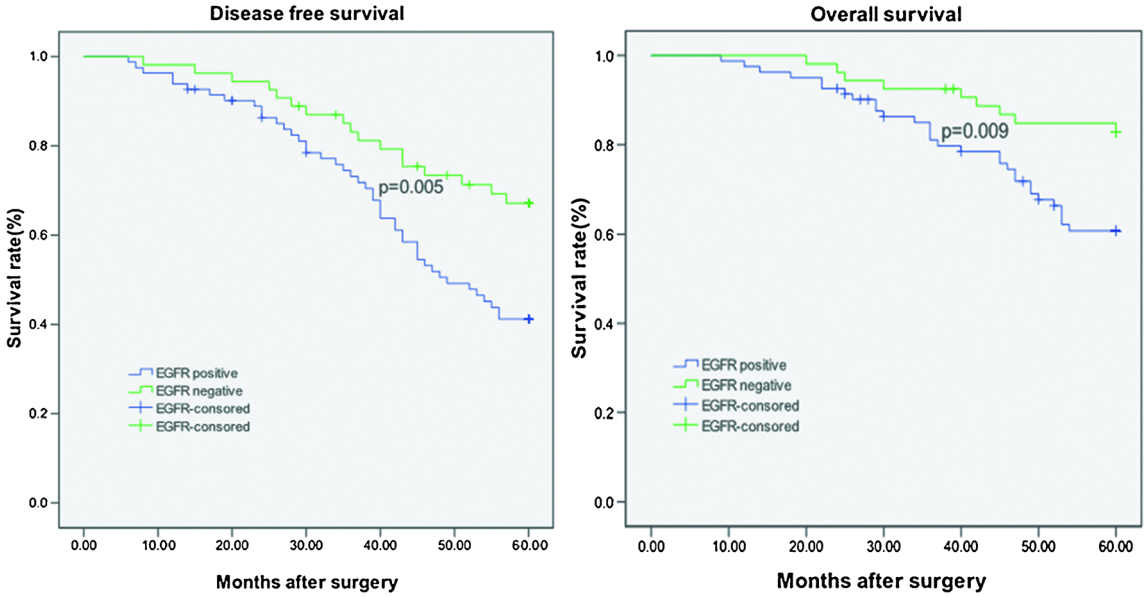

Fig. 2 The DFS and OS analyzed according to different EGFR expression status

the expression and clinical significance of EGFR and survivin were evaluated in 136 cases of TNBC. The results showed that EGFR and survivin expression could be predictive factors of TNBC. This study provides useful insights into the treatment and prognosis of breast cancer since it is based on a relatively large number of cases. The data presented in this study strongly indicate that EGFR and survivin overexpressions are involved in breast cancer progression and are independent prognostic factors of disease outcome.

Several promising targets have been evaluated, of which EGFR is the most extensively investigated target [26]. EGFR overexpression has been observed in many human cancers and found to be correlated with poor clinical prognosis, among them brain, head and neck, thyroid, lung, colorectal, urinary system, ovarian, as well as breast cancers [27–38]. Activation of the receptor with epidermal growth factor promotes proliferation and migration of tumor cells, thus facilitating the spread of cancer. Our results showed that EGFR protein was frequently expressed in TNBC and associated with important clinicopathological variables for disease outcome, such as menopausal status, lymph node metastasis, P53 expression, Ki-67 expression, and lymphatic vascular invasion. Also, EGFR was found to be an independent prognostic marker for DFS and OS. Various approaches to inhibit EGFR signaling have been investigated, with evaluation of mAbs against EGFR and EGFR-specific TKIs in phase III trials and

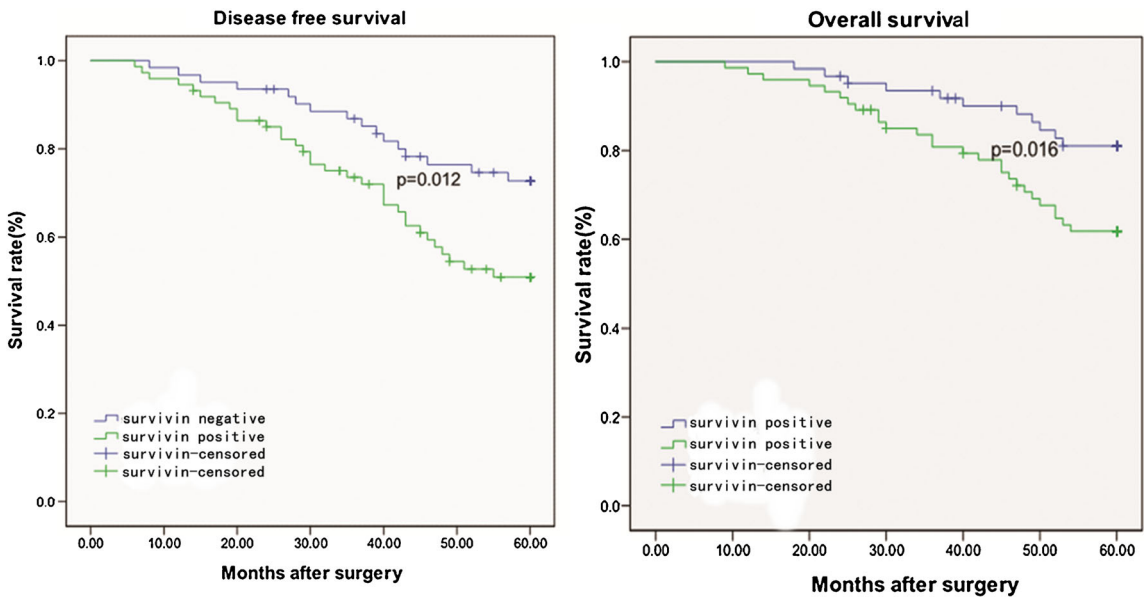

Fig. 3 The DFS and OS analyzed according to different survivin expression status

Table 3 Prognostic factors by multivariate analysis for triple-negative breast cancer patients

Parameters	Hazard ratio	*P* value	95 % CI
DFS			
Tumor size (>2 cm vs ≤2 cm)	1.587	0.018	1.081–2.330
Lymph node metastases	2.873	0.001	1.544–5.344
Tumor grade	1.914	0.005	1.218–3.007
EGFR	3.008	0.008	1.331–6.792
Survivin	1.573	0.016	1.087–2.277
OS			
Tumor size >2 cm	1.606	0.015	1.096–2.354
Lymph node metastases	2.915	0.001	1.553–5.471
Tumor grade	1.983	0.005	1.228–3.203
EGFR	3.151	0.007	1.374–7.226
Survivin	1.607	0.017	1.088–2.374

DFS disease-free survival, *EGFR* epidermal growth factor receptor, *OS* overall survival

the successful use of EGFR inhibition in the primary treatment of locally advanced head and neck squamous cell carcinoma (SCCHN) [39] and non-small cell lung cancer (NSCLC) [40, 41]. But these methods that inhibit EGFR signaling have not been so successful. Our results support the viewpoint that EGFR expression correlates with the aggressive behavior of TNBC and demonstrate that more efforts should be made to research the mechanism of EGFR influencing breast cancer, thus developing effective agents to cure breast cancer through the EGFR pathway.

Survivin is expressed in the G2-M phase of the cell cycle in a cell cycle-regulated manner and associates with microtubules of the mitotic spindle. Survivin has been known to be linked with human cancers, and its exceptional characteristics have been extensively investigated [12]. Survivin functions as an apoptosis inhibitor and a regulator of cell division. It is not detectable in most differentiated normal adult tissues but is expressed in a wide range of cancer tissues. Its expression in cancer has been correlated with poor prognosis, cancer progression, and drug resistance [42]. In the present study, we also found that survivin expression was associated with menopausal status, tumor size, lymph node metastasis, and poor survival in TNBC patients, but some studies indicate that there is no relationship between survivin expression and various clinicopathological factors [8, 19]. Recent results suggest that these discrepancies could be due to the use of various types of adjuvant therapy [43] or the different molecular subtypes.

Recent studies found a correlation in the molecular pathway of the expression of EGFR family molecules and the expression of survivin. A recent paper reported that an activated form of EGFR may elevate the levels of survivin and that an inactivation of the ErbB receptors may reduce the expression levels of survivin [44]. We did not intentionally investigate the relationship of these two markers when we designed this work. But interestingly, we found that survivin expression levels correlated with EGFR expression in the TNBC patients, and we believe that we are the first to demonstrate their correlation in TNBC. We found that almost all patients who were positive for survivin expression were positive for EGFR expression; only two patients were negative for the expression of EGFR. The shorter survival of survivin-positive patients with a negative EGFR might be due to other molecular characteristics associated with proliferation (e.g., platelet-derived growth factor receptor (PDGFR), methionine supply (Met)) and invasion [45]. But in 82 TNBC patients with positive EGFR, there were 22 patients who were negative for survivin. A possible explanation for this discrepancy is that the EGFR level does not reflect the activation level of EGFR. Thus, we suggest that EGFR in TNBC with a negative survivin might still be inactive and would become active to facilitate tumor progression. In the population with a negative survivin, the significant difference in survival between patients with positive and negative EGFR supports our hypothesis. The shorter survival of patients with positive EGFR might be due to the later activation of survivin. Thus, we propose that the determination of activated EGFR and the subsequent signaling pathway will be helpful in evaluating the prognostic value of EGFR and developing anti-EGFR therapies. The relationship of EGFR and survivin, and the mechanism of their interaction remain to be studied.

Our findings will be useful not only for understanding TNBC but also for effective clinical diagnosis. However, further studies are needed to clarify the mechanisms of the EGFR-mediated upregulation of survivin and the role of nuclear survivin expression in cancer of epithelial origin because these interactions may be important for potential therapeutic interventions in the future. In this series of operable TNBC treated with a multimodal approach, the nuclear expression of survivin was shown to be a strong prognostic indicator for a poor survival probability. Considering the clinical relevance of this finding, nuclear survivin expression should be further evaluated to select patients with an increased risk for disease recurrence. Additional studies of in vivo molecular signaling and/or cofactors of EGFR to induce expression of survivin in TNBC are likely to further highlight the advantage of combinational diagnosis with survivin and EGFR.

In conclusion, our study indicates that TNBC tends to display a more aggressive clinical course. Regardless of the underlying biological mechanism, EGFR and survivin expression status in combination proved to be of powerful prognostic predictive value to distinguish patients with a more biologically aggressive invasive breast carcinoma. Although our study has some limitations such as a retrospective design and relatively short follow-up period, the data provided by our study indicate that EGFR and survivin could serve as useful biomarkers to better determine TNBC prognosis.

New treatment strategies should be investigated for patients with triple-negative tumors as in HER2-positive tumors. Further studies will be needed to confirm the validity of our results and investigate the relationship of EGFR and survivin.

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Conflict of interest The authors have stated that they have no conflict of interest.

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