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Original Article

Expression of cleaved caspase-3 predicts good chemotherapy response but poor survival for patients with advanced primary triple-negative breast cancer

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Abstract: Objective: To assess cleaved caspase-3 (CC3), Ki-67, and E-cadherin (E-cad) expression in relation to chemotherapy response and prognosis of patients with advanced primary triple-negative breast cancer (TNBC). Methods: CC3 expression was detected immunohistochemically in 67 pre-chemotherapy biopsy samples. Ki67 and E-cad levels were obtained from patients' medical records. Results: CC3-positivity (N = 32; 47.8%) was associated with a higher first-line chemotherapy overall response rate (ORR; P = 0.028) and second-line chemotherapy clinical benefit rate (CBR; P = 0.033). The Ki-67 high-risk group (N = 51; 76.1%) exhibited a reduced second-line chemotherapy CBR (P = 0.024). The E-cad negative group (N = 25; 37.3%) exhibited a lower first-line chemotherapy ORR (P = 0.044) and CBR (P<0.001), and a lower second-line chemotherapy CBR (P = 0.020). CC3, Ki-67, and E-cad were significant predictors of third-line chemotherapy ORR or CBR. Similar numbers of chemotherapy cycles were completed by the CC3-positive and -negative groups. The Ki-67 high-risk and E-cad negative groups completed fewer second-line chemotherapy cycles (P = 0.038) and fewer first-line chemotherapy cycles, respectively (P = 0.001). Kaplan-Meier analyses identified worse outcomes for the CC3-positive, Ki-67 high-risk, and E-cad negative groups than for their corresponding comparison groups (P<0.05). Multivariate Cox regression analysis identified CC3 expression and an absence of E-cad expression as independent survival factors (P<0.05). Conclusions: Our CC3-positive group exhibited a better chemotherapy response, but a worse prognosis. The Ki-67 high-risk and E-cad negative groups exhibited both a worse chemotherapy response and worse prognosis.

Keywords: Triple-negative breast cancer, cleaved caspase-3, chemotherapy response, prognosis

Introduction

Triple-negative breast cancer (TNBC) is distinguished by an absence of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) expression. Consequently, patients with TNBC do not benefit from hormonal or trastuzumab-based therapies. To date, TNBC accounts for approximately 10-20% of all breast cancer cases diagnosed, and it more frequently affects younger patients and African-American women [1]. TNBC tumors are generally larger in size, are assigned a higher tumor grade, and exhibit a more aggressive phenotype compared with other types of breast cancer [2]. In addition, Farrah et al. reported that 14% of TNBC patients present with distant metastatic disease at the time of primary diagnosis [3]. In the absence of targeted therapies, chemotherapy remains the first line of treatment for TNBC patients. However, while TNBC is sensitive to chemotherapy, advanced TNBC has a very poor prognosis, with a median overall survival of 13.3 months [3]. Thus, there is an urgent need to identify new therapeutic targets for the treatment of primary advanced TNBC. In the meantime, the ability to enhance chemosensitivity and prolong patient survival would improve the prognosis of patients with primary advanced TNBC.

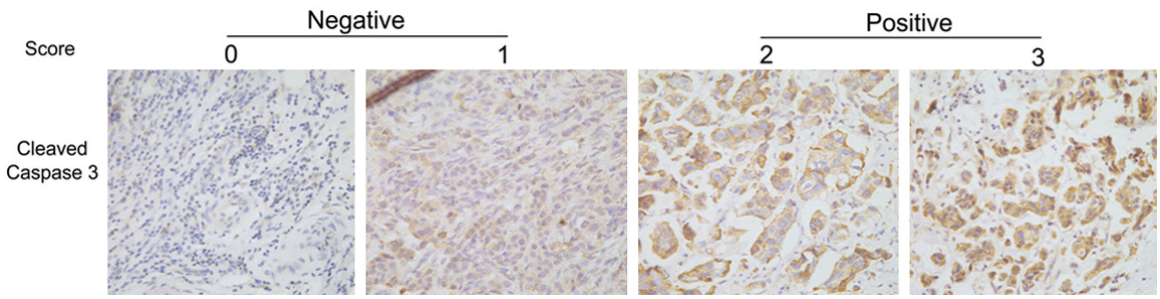

Figure 1. Representative IHC staining of TNBC biopsy tissues to demonstrate the scoring used to evaluate cleaved caspase-3 expression. Scores of 0 or 1 indicate negative staining and scores of 2 and 3 indicate positive staining.

Chemotherapeutic drugs mainly kill tumor cells by inducing apoptosis. Caspase-3, a central member of the cysteine-aspartic acid protease (caspase) family, has a dominant role in the apoptotic signaling pathway and in regulating cellular apoptosis. Correspondingly, caspase-3 plays an important role in the development of many types of cancers [4-6]. Branham et al. reported a very strong correlation between caspase-3 expression and pathological response to neoadjuvant therapy [7], while Végran et al. found that high levels of caspase-3 expression in locally advanced breast cancer led to greater sensitivity to neoadjuvant chemotherapy [8]. However, these studies had a limited period of chemotherapy and their TNBC subgroups were not analyzed separately.

Caspase-3 is initially translated into an inactive pro-enzyme form. Caspase-3 becomes activated when cells undergo apoptosis. Cleaved caspase-3 (CC3) is the active form of caspase-3 and it is able to proteolytically cleave and activate other caspases, as well as other relevant targets, in cells to regulate apoptosis. To date, there is no evidence that a synergistic relationship exists between caspase-3 and CC3. However, it has been reported by Zhou et al. that a higher percentage of TNBC cases are characterized by positive CC3 expression compared with non-TNBC cases [9]. Moreover, CC3 expression was found to significantly correlate with poor prognosis of breast cancer, including both the TNBC and non-TNBC subtypes [9]. It should be noted that the cases examined by Zhou et al. included stage I-III breast cancer patients who underwent radical surgery, and advanced primary TNBC cases were not included in their analysis.

Therefore, in this study, we have identified advanced primary cases of TNBC in which surgery was not performed in order to eliminate surgery bias to prognosis and to reduce the impact of postoperative preventive chemotherapy on chemosensitivity. Next, expression of CC3 was detected in biopsy samples available for these cases and these results were compared to chemosensitivity exhibited in response to three lines of chemotherapy treatment and patient prognosis. Since Ki-67 and E-cad have also rarely been studied in relation to primary advanced TNBC, expression levels of these targets were obtained from pathology reports and were also examined.

Materials and methods

Patients

The General Hospital of Shenyang Military Region (Shenyang, China) maintains a database of c-Stage IIIC (n = 13) and c-Stage IV (n = 54) patients. A search of this database was conducted according to the following inclusion criteria: 1) advanced TNBC presented at first diagnosis; 2) a pathologic diagnosis was made; and 3) radical or palliative resection was not performed. In addition, patients who did not undergo chemotherapy or those whose available samples were not sufficient to perform additional immunohistochemistry (IHC) analyses (e.g., for CC3) were excluded. Pathological samples were available for 67 advanced TNBC samples that were collected between January 2008 and December 2014. The samples were obtained from primary tumors or metastatic lymph nodes by aspiration biopsy and pathology confirmed a diagnosis of TNBC. Clinical stage was diagnosed according to color Doppler ultrasound, high-resolution computed tomography (HRCT), and/or positron-emission tomography (PET)/computed tomography (CT) and classified according to the guidelines of the National

Table 1. Clinicopathologic characteristics of advanced TNBC patients (n = 67)

Variable	CC3 expression		*P*-value	Ki-67 risk		*P*-value	E-cad expression		*P*-value
	Negative (n = 35)	Positive (n = 32)		≤ 30% (n = 16)	> 30% (n = 51)		Negative (n = 25)	Positive (n = 42)	
Age, y (mean ± SD)	57.7 ± 11.7	55.7 ± 9.7	0.439	58.8 ± 12.4	56.1 ± 10.2	0.379	56.2 ± 9.6	57.1 ± 11.5	0.758
Menopause									
Yes	25	21	0.793	13	33	0.354	19	27	0.417
No	10	11		3	18		6	15	
T stage	26	26	0.556						
T1 + T2	9	6		13	39	1.000	21	31	0.381
T3 + T4				3	12		4	11	
N stage									
N0 + N1	12	12	0.804	6	18	1.000	5	19	0.064
N2 + N3	23	20		10	33		20	23	
TNM stage									
IIIC	9	4	0.223	4	9	0.493	4	9	0.753
IV	26	28		12	42		21	33	
Grade									
2	2	1	1.000	0	3	1.000	2	1	0.551
3	33	31		16	48		23	41	
CC3 expression									
Negative	N/A	N/A	N/A	13	22	0.010	10	25	0.138
Positive	N/A	N/A		3	29		15	17	
Ki-67 risk (mean ± SD)	55.1 ± 27.9%	68.8 ± 21.3%	0.028	N/A	N/A	N/A	69.2 ± 23.5%	57.1 ± 26.2%	0.063
Ki-67 risk									
≤ 30%	13	3	0.010	N/A	N/A	N/A	4	12	0.375
> 30%	22	29		N/A	N/A		21	30	
E-cad									
Negative	10	15	0.138	4	21	0.375	N/A	N/A	N/A
Positive	25	17		12	30		N/A	N/A	
P53									
Negative	11	7	0.420	7	11	0.108	8	10	0.571
Positive	24	25		9	40		17	32	

CC3: cleaved caspase-3; SD: standard deviation; TNM: tumor, node, metastasis.

Table 2. Patient response to first-line chemotherapy (n = 67)

Variable	CR+PR	SD+PD	*P*-value	CR+PR+SD	SD	*P*-value
CC3 expression						
Negative	11	24	0.028	28	7	1.000
Positive	19	13		26	6	
Ki-67 risk						
≤ 30%	4	12	0.088	15	1	0.165
> 30%	26	25		39	12	
E-cad						
Negative	7	18	0.044	14	11	<0.001
Positive	23	19		40	2	

CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease.

Comprehensive Cancer Network (NCCN; Version 1.2017). The medical records corresponding to the selected TNBC samples were reviewed separately. Approval for this study was obtained from the Ethics Committee of China Medical University and each patient provided written informed consent.

Chemotherapy

First-line chemotherapy was anthracycline-based and was given with or without paclitaxel at our treatment center. Second-line chemotherapy was cisplatin-based as a single or combined treatment, while third-line chemotherapy was gemcitabine-based as a single or combined treatment or included other chemotherapeutics. Chemotherapy response was classified as complete (CR), partial (PR), or as stable disease (SD) versus progressive disease (PD). Overall response

Table 3. Patient response to second-line chemotherapy (n = 54)

Variable	CR+PR	SD+PD	*P*-value	CR+PR+SD	PD	*P*-value
CC3 expression						
Negative	5	23	0.218	11	17	0.033
Positive	9	17		18	8	
Ki-67 risk						
≤ 30%	3	9	1.000	10	2	0.024
> 30%	11	31		19	23	
E-cad						
Negative	4	13	1.000	5	12	0.020
Positive	10	27		24	13	

CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease.

Table 4. Patient response to third-line chemotherapy (n = 31)

Variable	CR+PR	SD+PD	*P*-value	CR+PR+SD	PD	*P*-value
CC3 expression						
Negative	1	16	0.576	5	12	1.000
Positive	2	12		5	9	
Ki-67 risk						
≤ 30%	2	9	0.281	5	6	0.423
> 30%	1	19		5	15	
E-cad						
Negative	0	6	1.000	4	2	0.067
Positive	3	22		6	19	

CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease.

rate (ORR) was calculated as the sum of CR + PR. Clinical benefit rate (CBR) was calculated as the sum of CR + PR + SD. Primary tumors were used as observed indicators for chemotherapy response. However, when a new metastatic lesion was diagnosed, the response was directly considered to be PD.

Immunohistochemistry

Formalin-fixed and paraffin-embedded tissue specimens were cut into 3-μm-thick sections. To detect CC3, the sections were deparaffinized in xylene and rehydrated in a graded alcohol series. The sections were subsequently heated in citrate buffer (pH 6.0) for 30 min at 93°C in a microwave oven for antigen retrieval, then were incubated in 20% normal serum) for 50 min at room temperature. Sections were incubated with an anti-active caspase-3 antibody (ab2302; Abcam) at 4°C overnight. Sections were incubated with phosphate buffered saline (PBS) instead of the primary antibody as a negative control. The next day, sections were rinsed three times in PBS (5 min each) and then were incubated with an appropriate secondary antibody (ab6112; Abcam) for 30 min at room temperature. After the sections were washed three times with PBS, the sections were incubated with a 3,3'-diaminobenzidine solution for up to 10 min to allow color development. The stained tissue sections were reviewed and scored independently by two experienced pathologists who were blinded to the diagnosis and clinical assessment of each sample. If a disagreement occurred, the stained sections were re-evaluated to reach a consensus. A negative result was defined as an absence of staining, or staining that represented less than 10% of the tumor cells present in a sample. This standard was applied to both CC3 and E-cad stainings. The latter data were obtained from pathology reports, as were Ki-67 levels. Mean percentage of nuclear positivity was evaluated with manual counting and it was performed by two independent pathologists.

Statistical analyses

Statistical analyses were performed by using Statistical Package for the Social Sciences software (SPSS version 22, IBM, Armonk, NY, USA). Student's *t*-test or the Wilcoxon Rank-Sum tests were used to compare continuous variables, while categorical variables were compared with the χ^2 test or Fisher's exact test. Survival curves were calculated according to the Kaplan-Meier method. Survival data were evaluated with univariate and multivariate Cox regression analyses (Forward: LR). *P*-values less than 0.05 were considered significant in all analyses performed.

Results

Protein expression and clinicopathological features

A total of 67 tissue samples that were previously collected by aspiration biopsy from a

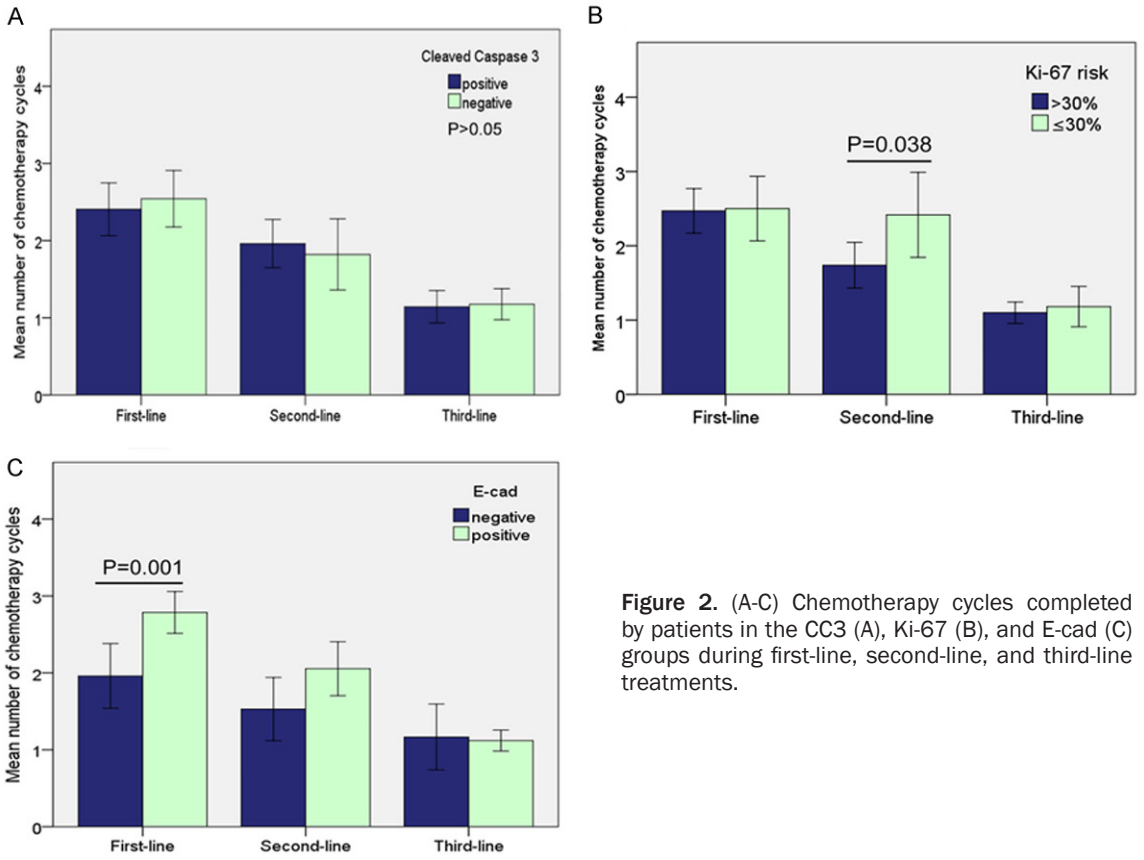

Figure 2. (A-C) Chemotherapy cycles completed by patients in the CC3 (A), Ki-67 (B), and E-cad (C) groups during first-line, second-line, and third-line treatments.

cohort of patients with advanced primary TNBC (mean age, 56.7 years) were available for analysis. Forty-six (68.7%) of these patients were in menopause and all had their diagnosis of TNBC confirmed with pathology.

Expression of Ki-67 and E-cad were previously characterized for these samples as reported in their pathology reports. For this study, the available tissue blocks were further sectioned and subjected to IHC to detect expression of CC3. Thirty-three samples (47.8%) showed positive staining for CC3 (**Figure 1**), 51 samples (76.1%) had high-risk levels of Ki-67 (risk > 30%), and 25 samples (37.3%) were negative for E-cad. Expression of these proteins, and p53, were analyzed in relation to patient age, menopause, T stage, N stage, TNM stage, and tumor grade (**Table 1**). A positive correlation was only observed between CC3 expression and high-risk levels of Ki-67 (P = 0.010).

Protein expression and chemotherapy

All patients in our cohort underwent 2.48 (± 1.01) cycles of first-line chemotherapy and their ORR and CBR were 44.8% and 80.6%, respectively. A total of 54 patients (80.6%) underwent additional cycles of second-line chemotherapy (1.89 ± 1.00), and their ORR and CBR were 25.9% and 53.7%, respectively. Finally, 31 patients (46.3%) underwent 1.13 (± 0.14) cycles of third-line chemotherapy, and their ORR and CBR were 9.7% and 32.3%, respectively.

Next, we analyzed chemotherapy response according to expression levels of CC3, Ki-67, and E-cad (**Tables 2-4** and **Figure 2A-C**). The CC3-positive samples were associated with a higher ORR to first-line chemotherapy (P = 0.028) and a higher CBR to second-line chemotherapy (P = 0.033). Meanwhile, the ORR and CBR were similar in response to third-line chemotherapy. Both the CC3-positive and CC3-negative groups completed similar numbers of cycles for the first-, second-, and third-line chemotherapy treatments. The Ki-67 high-risk group had a lower CBR in response to second-line chemotherapy (P = 0.024), while similar responses were observed following the first-

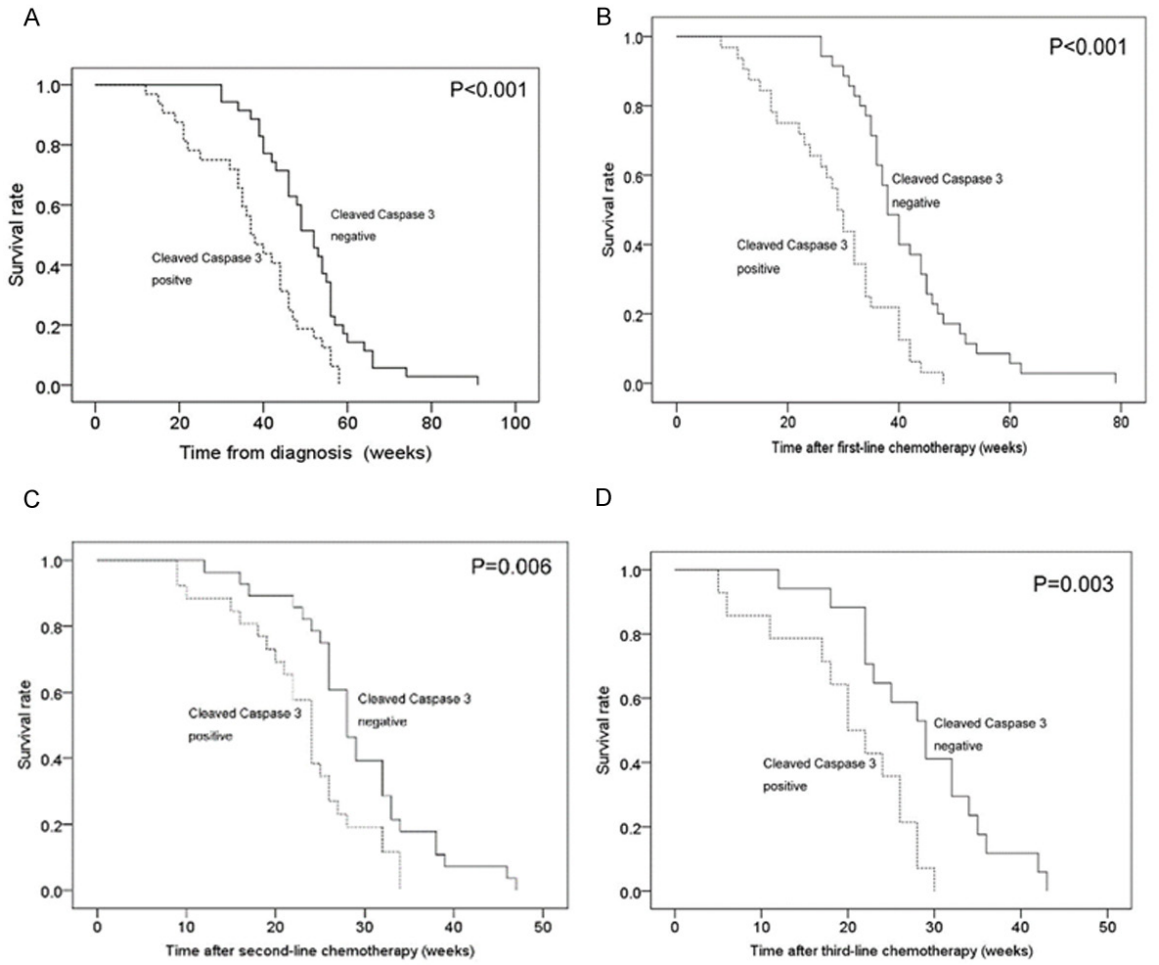

Figure 3. A-D. Association between cleaved caspase-3 expression and patient survival in different treatment phases.

and third-line treatments. Regarding second-line chemotherapy, the Ki-67 high-risk group completed fewer cycles (P = 0.038), yet similar numbers of cycles were completed in their first-line and third-line chemotherapy treatments. The E-cad-negative group had a lower ORR (P = 0.044) and CBR (P<0.001) to first-line chemotherapy, a lower CBR (P = 0.020) to second-line chemotherapy, and a similar response to third-line chemotherapy. In addition, the E-cad-negative group had fewer cycles during their first-line treatment, and a similar number of cycles for their second-line and third-line chemotherapy treatments.

Protein expression and survival

The survival time for the patients examined ranged from 12 weeks to 91 weeks (median, 46 weeks). To further understand whether the chemotherapy responses associated with the different protein expression profiles affected tumor progression and survival, overall survival and survival according to treatment regimen were analyzed. The better chemotherapy response of the CC3-positive group did not correspond with prolonged survival time (**Figure 3A-D**). In fact, the CC3-positive group exhibited a worse outcome compared to the negative group (P<0.001), and CC3 positivity predicted worse survival at each treatment phase (all, P<0.05). A similar method was used to analyze the Ki-67 and E-cad groups. The Ki-67 high-risk group exhibited worse overall survival (P = 0.018) and worse survival after first-line chemotherapy (P = 0.005) (**Figure 4A-D**). In contrast, survival after second-line and third-line chemotherapy treatments did not significantly differ (both, P > 0.05). When we considered the chemotherapy response and cycles of the

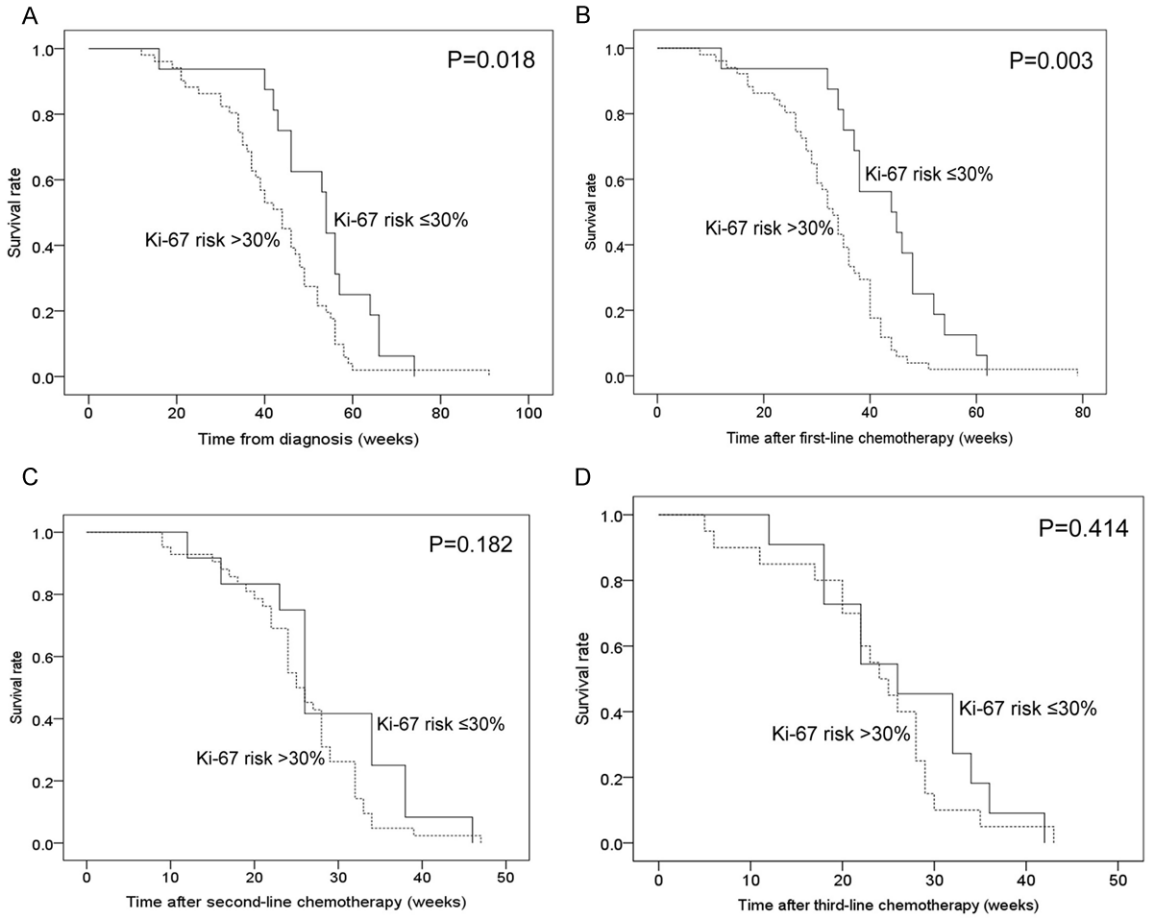

Figure 4. A-D. Association of Ki-67 expression with patient survival in different treatment phases.

Ki-67 groups, the Ki-67 high-risk group was found to have a poor response to second-line chemotherapy and fewer chemotherapy cycles were completed. It is possible that the latter condition was the reason for the worse outcome of the Ki-67 high-risk group. Similar to the Ki-67 group, the E-cad negative group exhibited worse overall survival (P<0.001) and worse survival after first-line chemotherapy (P = 0.005) (**Figure 5A-D**). Meanwhile, survival after the second-line and third-line chemotherapy treatments did not significantly differ (both, P > 0.05). When we considered the chemotherapy response and cycles of the E-cad expression groups, the E-cad-negative group exhibited a poor response to first-line and second-line chemotherapies and fewer chemotherapy cycles were completed for the first-line treatment (P = 0.001). A tendency for fewer cycles to be completed in the second-line treatment was also observed (P = 0.074), and this may account for the shorter survival time for the E-cad negative group.

Protein expression and independent prognosis factors

We further examined whether expression levels of CC3, Ki67, and E-cad represent independent factors for patient survival with univariate and multivariate Cox regression analyses. In the former, TNM stage and expression levels of CC3, Ki-67, and E-cad were associated with overall survival (all P<0.05) (**Table 5**). Meanwhile, in the multivariate Cox regression analysis, TNM stage and expression levels of CC3 and E-cad were identified as independent factors for predicting survival (all P<0.05).

Discussion

In the present study, expression of CC3 was detected by IHC in 67 biopsy samples that were

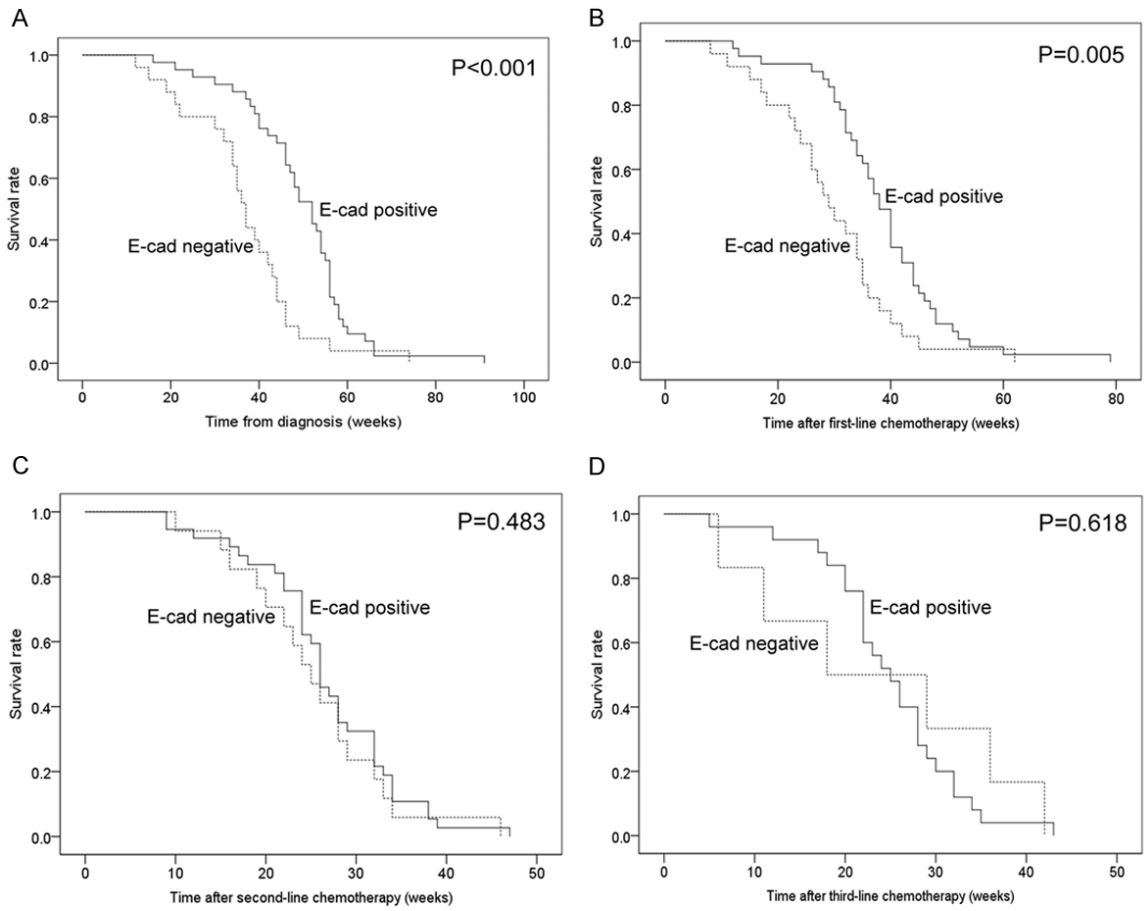

Figure 5. A-D. Association of E-cad expression with patient survival in different treatment phases.

obtained from advanced primary TNBCs. In addition, expression of Ki-67 and E-cad were obtained from the pathology reports for each sample, while clinical characteristics, chemotherapy response, and outcome were obtained from patient medical records. The CC3-positive group was associated with a better chemotherapy response, yet it suffered a worse prognosis. Meanwhile, the Ki-67 high-risk and E-cad-negative groups were associated with a worse chemotherapy response and a worse prognosis. Multivariate Cox regression analysis further identified CC3 and E-cad expression levels as independent prognostic factors for patients with advanced primary TNBC.

Caspase-3 is an important protein in the apoptosis pathway. It is the major executioner caspase during the demolition phase of apoptosis [10], and higher levels of caspase-3 expression have characterized many malignancies [10-12]. However, to date, the role of caspase-3 in breast cancer remains controversial [13-15], particularly the relationship between caspase-3 expression and breast cancer prognosis [8, 9, 16, 17]. CC3 (17 kDa) is the activated form of caspase-3, and to our knowledge, the present study is the first to detect it in advanced primary TNBC tissues. CC3 can initiate protein degradation and irreversible cell apoptosis by cleaving substrate proteins such as poly-ADP-ribose polymerase (PARP) [18]. The PARP family of proteins mediate DNA repair and maintenance of cell stability [19]. Thus, during anthracycline- and platinum-based chemotherapy treatments, direct or indirect DNA damage that is generated is counteracted by PARP proteins which repair DNA damage [19]. However, when PARP proteins are cleaved by CC3, they are deactivated and cell apoptosis is accelerated. Consequently, PARP inhibitors have been developed for clinical therapy for many years (including tricyclicindoles, benzimidazoles, and pthalazinones). PARP inhibition has also been found to potentiate the clinical efficacy of alkylating agents (temozolomide), topoisomerase inhibi-

Table 5. Univariate and multivariate Cox regression analysis of OS for all patients

Variables	Univariate		Multivariate	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Age				
≤ 50 y				
> 50 y	0.876 (0.523–1.466)	0.614	N/A	N/A
Menopause				
Yes				
No	0.752 (0.440–1.287)	0.299	N/A	N/A
T stage				
T1+T2				
T3+T4	0.945 (0.525–1.702)	0.850	N/A	N/A
N stage				
N0+N1				
N2+N3	0.796 (0.474–1.335)	0.386	N/A	N/A
TNM stage				
IIIC				
IV	2.404 (1.261–4.584)	0.008	2.021 (1.061–3.849)	0.032
Grade				
G2				
G3	0.398 (0.120–1.322)	0.133	N/A	N/A
CC3				
Negative				
Positive	2.472 (1.477–4.139)	0.001	2.658 (1.563–4.522)	<0.001
Ki-67 risk				
≤ 30%				
> 30%	1.940 (1.084–3.473)	0.026	N/A	N/A
E-cad				
Negative				
Positive	0.404 (0.241–0.680)	0.001	0.402 (0.233–0.695)	0.001
P53				
Negative	1.263 (0.730–2.185)	0.404	N/A	N/A
Positive				

OS: overall survival; HR: hazard ratio; CI: confidence interval; TNM: tumor, node, metastasis; N/A: not available.

tors (irinotecan and topotecan), and ionizing radiation [20]. With PARP proteins being one of the most important substrates of CC3, we hypothesize that CC3 mediates a similar function as PARP inhibitors, and this may account for the better chemotherapy response of the CC3-positive group in the present study. However, CC3 expression was not beneficial for survival despite being associated with a better chemotherapy response. Actually, the CC3-positive group had worse survival than CC3-negative patients for unknown reasons. Expression of CC3 was also associated with high-risk Ki-67 levels, which indicates that the CC3-positive tissues are characterized by greater malignancy and a higher appreciation rate. Therefore, we hypothesize that expression of CC3 is not a cause of tumorigenesis, but rather is a consequence of tumorigenesis. For example, greater malignancy is associated with more active tumor cell apoptosis signaling, and this could result in higher expression of CC3. However, it remains unclear why tumor cells fail to undergo apoptosis or suffer low apoptosis efficiency. Thus, further studies are needed to investigate whether expression of CC3 reduces the effect of PARP inhibitors, although the results of the present study support the identification of enhanced CC3 expression as a new therapeutic target.

Ki-67 has been considered a prognostic marker in breast cancer by several international guidelines [21, 22]. Moreover, TNBC has been classified into two Ki-67 subtypes, and each have different survival characteristics [23]. However, to our knowledge, very few studies have focused on the value of Ki-67 in chemotherapy response and prognosis of patients with advanced primary TNBC. In our cohort, the Ki-67 high-risk group exhibited a worse chemotherapy response, fewer chemotherapy cycles were completed, and worse survival was observed. Because there is no clear molecular evidence that Ki-67 is associated with chemosensitivity, we hypothesize that low-risk levels of Ki-67 do not correlate with a better chemotherapy response, while high-risk levels of Ki-67 lead to an enhanced cell proliferation rate and accelerated tumor progression. In this study, the chemotherapy protocol had to be modified or discontinued due to tumor progres-

sion, and this may account for the poor chemotherapy response of the Ki-67 high-risk group.

E-cad has important roles in maintaining cell polarity and integrity [24], and also in tumorigenesis, tumor progression, invasion, and metastasis [25]. During the epithelial-to-mesenchymal transition in cancer cells, downregulation of E-cad expression is a key aspect. In a study by Liu et al. [7], absence of E-cad expression was found to characterize TNBC cases more often than non-TNBC cases. However, until now, the expression and role of E-cad in advanced primary TNBC has remained unclear. Similar to the Ki-67 group, the E-cad negative group in the present study was associated with rapid tumor progression, and this resulted in fewer chemotherapy cycles and worse survival.

It should be noted that there were limitations associated with the present study. First, although all of the patients received similar chemotherapy drugs, many different chemotherapy protocols were employed and this potentially introduced treatment effect bias. Second, use of other adjuvant therapies, including radiotherapy, targeted therapy, immunotherapy, and traditional Chinese medicine therapy, may have also contributed to a bias to treatment effect. Third, IHC assays to detect Ki-67, E-cad, and P53, as well as ER, PR, and HER2, were performed and evaluated by different pathologists in the pathology department of our hospital. Thus, this may represent another source of bias.

In the present study, CC3 expression correlated with a better chemotherapy response, yet a poorer prognosis, in patients with advanced primary TNBC. CC3 was also identified as a potential independent prognostic factor and new therapeutic target for advanced primary TNBC. It is anticipated that further study of this activated protein may provide valuable insight into chemotherapy resistance and may also lead to the identification of additional therapeutic targets.

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Disclosure of conflict of interest

None.

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