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ORIGINAL ARTICLE

Potential candidate biomarkers for heterogeneity in triple-negative breast cancer (TNBC)

Eun Yoon Cho · Myung Hee Chang · Yoon La Choi · Jeong Eon Lee · Seok Jin Nam · Jung-Hyun Yang · Yeon Hee Park · Jin Seok Ahn · Young-Hyuck Im

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Abstract
Purpose Triple-negative breast cancer (TNBC) exhibits a distinct pattern of recurrence characterized by a rapidly rising rate in the first 2 years with a peak at 2–3 years, followed by a decline over the next 5 years. However, some TNBC patients exhibit indolent clinical behavior. The aim of this study was to investigate clinical characteristics and outcomes of metastatic TNBCs. In addition, we were to find out the marker which could divide TNBCs into a few subgroups according to different clinical features.
Methods We retrospectively analyzed the clinicopathologic characteristics and outcomes of patients with metastatic TNBC who received palliative treatment between 1999 and 2007.
Results The median relapse-free survival (RFS) and overall survival (OS) of 152 patients were 24 and 20 months, respectively. Divided TNBCs based on a RFS of 36 months, the patients with a RFS of $\geq$36 months had a better disease control rate (DCR), progression-free survival (PFS), and OS than those with a RFS of <3 years. Cox-regression multivariate analysis for RFS and OS revealed EGFR positivity and a low BRCA1 score as an independent risk factor for OS.
Conclusions The patients with a RFS $\geq$ 3 years had a significantly better DCR, PFS, and OS than patients who had a RFS < 3 years. BRCA1 and EGFR expression may be candidate determinants to distinguish RFS. A prospective clinical trial for different therapeutic strategies is needed for each subgroup.

Keywords Triple-negative breast cancer · Relapse-free survival · BRCA1 · EGFR

Introduction

Breast cancer represents a heterogeneous group of diseases that vary in morphology, biologic behavior, and response to therapy. Gene expression profiles using DNA microarrays have shown at least 4–5 distinct breast cancer subtypes, such as luminal A, luminal B, basal-like, human epidermal growth factor receptor-2 (HER2), and normal breast-like [1]. Basal-like breast cancer (BLBC) is characterized by constitutive expression of genes found in normal basal/myoepithelial cells of the breast, including basal cytokeratins (CK5/6, CK14, and CK17), vimentin, P-cadherin, and p63 [2]. Triple-negative breast cancer (TNBC) is now commonly used to define breast cancers that are estrogen receptor (ER) negative, progesterone receptor (PgR) negative, and HER2 negative using the clinical assays for these biomarkers [2–4] BLBC is similar to TNBC because almost all BLBCs do not express ER, PgR, and HER2. However, BLBC refers to a molecular phenotype defined by an intrinsic gene set identified using mRNA gene expression profiling [5–7]. Furthermore,

Eun Yoon Cho and Myung Hee Chang contributed equally in this study.

M. H. Chang · Y. H. Park · J. S. Ahn · Y.-H. Im (✉)
Division of Hematology-Oncology, Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50 Irwon-dong, Gangnam-gu, Seoul 135-710, Korea
e-mail: imyh00@skku.edu

E. Y. Cho · Y. L. Choi
Department of Pathology, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea

J. E. Lee · S. J. Nam · J.-H. Yang
Department of Surgery, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea

analysis of microarray-based expression profiling suggests that in addition to the basal-like cluster, the triple-negative group also encompasses tumors within the normal-like breast cancer molecular subtype [8–13]. Recent evidence has shown that TNBC is a heterogeneous group of tumors and those exhibiting a basal-like phenotype comprise only a subset of these cancers. Similarly, BLBCs, as defined by microarray profiling, also show significant heterogeneity according to the expression of different gene modules identified using a clustering approach [14–16]. Although BRCA1-associated tumors, BLBC, and TNBC overlap, they are neither synonymous nor identical.

TNBCs comprise 10–15% of all breast cancers [17–21] and are of pivotal clinical importance given the lack of effective therapeutic options, in spite of worse outcomes to conventional treatment [2, 8, 23]. The aggressive clinical course, poor prognosis, and lack of therapeutic options for this type of tumor have intensified current interest in this patient group.

TNBCs carry a distinct pattern of relapse, which was not found in HR-positive tumors [8–10, 17, 22, 23]. This relapse pattern is characterized by a rapidly rising rate in the first 2 years following diagnosis, a peak at 2–3 years, followed by a decline in recurrence risk over the next 5 years. However, some of the TNBC patients show indolent clinical behavior associated with a good clinical outcome [11]. This suggests that TNBC may be a heterogeneous group comprising subtypes with different biologic features and clinical outcomes.

The aims of this study were to (1) analyze the clinicopathologic features of TNBC according to relapse pattern to elucidate different biologic behaviors and (2) identify potential candidate biomarkers for the different TNBC subgroups and the corresponding therapeutic outcomes.

Methods

Patients and data collection

Between 1997 and 2007, 857 patients with metastatic breast cancer (MBC) received palliative chemotherapy, hormonal therapy, and/or targeted therapy at Samsung Medical Center (SMC). All pathologic specimens were by reviewed two experienced pathologists, who determined the receptor status of the ER and HER2 using immunohistochemical (IHC) stainings. Of these, 173 patients were identified as ER/PR- and HER2-negative TNBCs. We retrospectively obtained patients' medical records, including demographic characteristics and clinical follow-up data. The patients were followed up for a median of 73.5 months (range, 24.2–120.0 months). This study was approved by the Samsung Medical Center Institutional Review Board.

Immunohistochemistry (IHC)

Expression of ER, PgR, and HER2 was analyzed immunohistochemically using the formalin-fixed, paraffin-embedded tumor tissues. ER and PgR negativity was defined as an Allred score from 0–2 out of 8 using antibodies to the ER (Immunotech, France) and PR (Novocastra, UK). HER2 status was evaluated using an antibody (DAKO, USA) and/or fluorescence in situ hybridization (FISH). Grades 0 and 1 for HER2 by IHC were defined as a negative result and grade 3 as a positive result. Amplification of HER2 was confirmed by FISH if HER2 was rated 2+ by IHC. 'Triple negativity' was defined as a lack of ER, PgR, and HER2 expression. To identify the potential candidate markers for the different TNBC subgroups and the corresponding therapeutic outcomes, we evaluated protein expressions of CK 14/17, EGFR, c-Kit, caveolin 1, VEGF, p53, and BRCA1 in their tumor tissues as well as those of ER, PgR, and HER2. The dilution concentration, sources, and cutoff values of primary antibodies used for IHCs other than ER, PgR, and HER2 are shown in Table 1. The slides were independently assessed by two experienced pathologists without knowledge of the clinical outcome. The intensity and extent of the IHC staining was graded on a semi-quantitative scale from 0–3 as follows: 0 = no staining; 1 = weak staining; 2 = moderate staining; and 3 = strong staining. Nuclear and cytoplasmic stainings were found in BRCA1 staining. The area of the most intense staining was graded on a scale from 0–4 as follows: 0 = no staining; 1 = staining of 0–10% of tumor cells; 2 = staining of 10–25% of tumor cells; 3 = staining of 25–50% of tumor cells; and 4 = staining of >50% of tumor cells. A score was calculated by multiplying the intensity and grades of stained areas for other IHC stainings for VEGF, BRCA1, caveolin 1, and c-Kit. The cutoff score for each IHC was found in this study. EGFR, CK14, and CK17 IHC stainings were interpreted as 'positive' or 'negative' according to the degree of membrane staining (cutoff value, 10%). p53 and Ki-67 stainings were interpreted as grades with the area of the most intense staining.

Table 1 Antibodies for use of immunohistochemical stainings

Antibody	Dilution	Source	Cutoff (%)
EGFR	1:10	Novocastra, UK	10
CK14	1:100	DAKO, Santa Barbara, CA	10
CK17	1:20	DAKO, Santa Barbara, CA	10
C-kit	1:200	Novocastra, UK	0
CAV1	1:150	DAKO, Santa Barbara, CA	0
VEGF	1:10	Invitrogen, Carlsbad, CA	0
P53	1:50	Invitrogen, Carlsbad, CA	5
BRCA1	1:150	Abcam, Cambridge, MA	0

EGFR epidermal growth factor receptor, *CK14* cytokeratin 14, *CK17* cytokeratin 17, *C-kit* CD117, *CAV1* caveolin 1, *VEGF* vascular endothelial growth factor

Statistical analysis

Relapse-free survival (RFS) was measured from the day of curative surgery to the first day of documented recurrence. Progression-free survival (PFS) was measured from the first day of the first-line palliative chemotherapy after recurrence to disease progression or death. Overall survival (OS) was measured from the day of the first-line chemotherapy to death or the last follow-up day.

In the univariate analysis, independent sample *t* tests and χ^2 tests were used for continuous and categorical variables, respectively. The Kaplan–Meier product-limit method was used to estimate the RFS, PFS, and OS. The survival rates were compared using the log-rank test. Multivariate analysis of the independent prognostic factors for survival was performed using the backward stepwise (likelihood-ratio statistics based on the conditional parameter estimate) method of the Cox proportional hazard regression model with 95% confidence intervals (CIs).

REMARK guidelines

In reporting our study, we have adhered to the guidelines in 2005 of an important methodology paper entitled "Reporting recommendations for tumor marker prognostic studies (REMARK guidelines)" [24].

Results

Patients' cohort and characteristics

Figure 1 shows the patient cohort of the study. Of 857 patients with metastatic breast cancer who received palliative management at Samsung Medical Center between 1999 and 2007, 173 TNBCs were identified. Of the 173 patients, the number of relapsed patients after curative surgery was 152, and the remaining 21 patients had initial metastatic disease. Our final cohort included 152 recurrent patients with TNBC. The median age was 45 years (range, 21–81 years), and the common metastatic sites were lung (46.2%), bone (40.5%), liver (17.9%), pleura (15.4%), soft tissue (14.5%), and brain (12%).

Relapse patterns

Most of the patients who underwent surgery for breast cancer were followed regularly, including a history and physical examination at 3–6 months and mammography at 1-year intervals for 5 years after curative resection of breast cancer. A medical history and physical examination once a year and annual mammography were performed. In cases of dense breasts, ultrasonography of the breast was added to yearly mammography. Other imaging studies such as ultrasonography of upper abdomen, computed tomography of chest and/or abdomino-pelvic scan, PET–CT scan, bone scan, and blood test, including tumor markers (CEA and CA 15–3), were performed as needed. Figure 2 shows the percentage of the relapsed patients at 1-year intervals for a median follow-up duration of 71 months. The rate of relapse was calculated every 12 months. The peak of relapse occurred 24 months after surgery. Because most of the relapses occurred within 36 months following surgery, we divided 152 patients into 2 groups according to RFS (<36 months vs. ≥36 months) and compared their clinical characteristics; one group who showed early relapse within 36 months ($n = 118$ [78%]) and the other group who had relapsed 36 months or later ($n = 34$ [22%]) (Fig. 2).

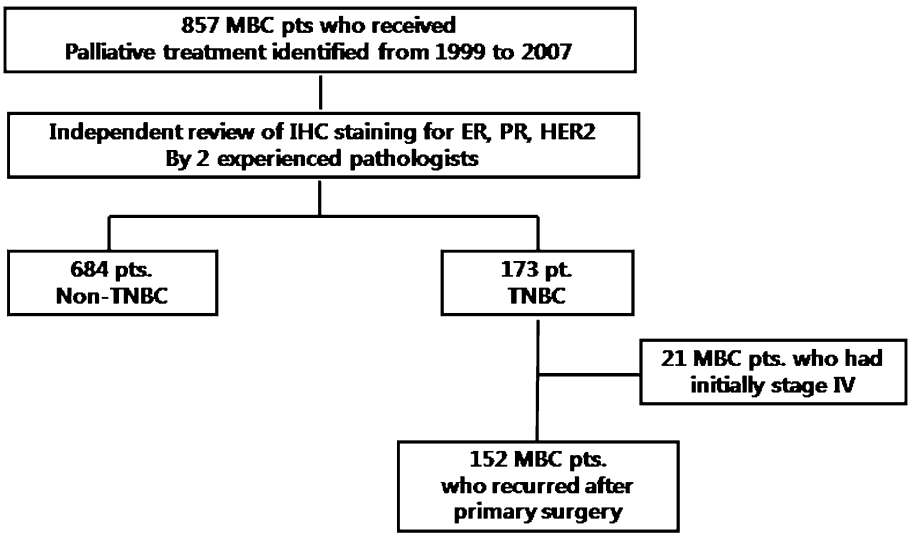

Fig. 1 Patients' cohort

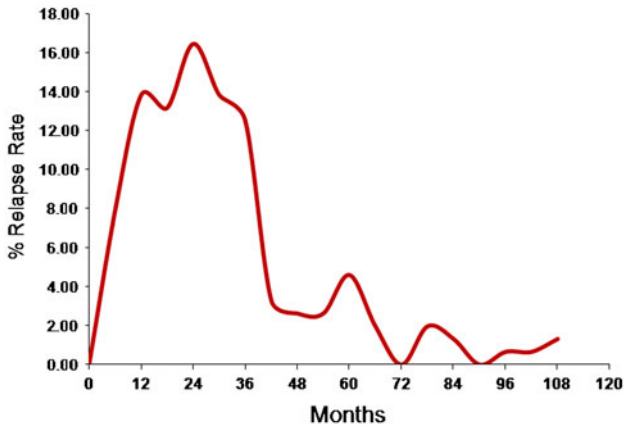

Fig. 2 Relapse Rates of the patients with TNBC after surgery at 36 months interval ($n = 152$)

Clinicopathologic characteristics according to RFS (Table 2)

The median RFS of 152 relapsed patients after curative surgery was 23.6 months (95% CI, 20.4–26.8 months). There were significant differences in terms of nuclear grade (high grade 69.1% vs. 50.0%, $P = 0.037$) and the incidence of brain metastases at the time of first metastasis (16.1% vs. 2.9%, $P = 0.047$) between the patients who had a RFS of ≤36 months and the patients who had a RFS > 36 months (Table 2). Most of the patients with a RFS ≥ 36 months received platinum plus taxane or doxorubicin-containing combination chemotherapy as first-line palliative chemotherapy, but statistical significance was not reached. The TNBC patients who had a RFS of ≥36 months showed a better response rate (37% vs. 65%, $P = 0.007$) and a better disease control rate (DCR; 55% vs. 77%, $P = 0.022$) to first-line palliative chemotherapy. The patients who had a RFS of ≥36 months had a better PFS to first-line palliative chemotherapy (3.6 vs. 7.7 months, $P = 0.0001$) and a better OS (16.7 vs. 41.3 months, $P = 0.0004$) than those with a RFS < 36 months (Fig. 3).

Correlation between IHC expression and clinical outcomes

Eighty-eight patients had archival breast tumor samples available for additional IHC studies of BRCA1, caveolin 1 (CAV1), EGFR, CK14, CK17, c-Kit, VEGF, Ki-67, and p53 (Fig. 4). Basal markers of EGFR, CK17, and CK14 were positive in 70, 53, and 43% of 88 TNBC patients, respectively. The association between the expression of these candidate gene proteins and clinical outcomes according to RFS, as measured by IHC, is listed in Table 3. Among 8 IHC studies, a low VEGF score ($P = 0.041$), high Ki67 index ($P = 0.001$), positive EGFR ($P = 0.005$), and positive CK14 ($P = 0.001$) were shown more commonly in TNBC patients with a RFS < 36 months with statistical significance by a χ^2 test. A low BRCA1 score and CK17 positivity were associated with a RFS < 36 months, with marginal significance (Table 3).

Figure 5a and b show Kaplan–Meier survival curves of RFS between the patients who were EGFR positive and EGFR negative (median RFS 20.2 vs. 26.1 months, $P = 0.050$, Fig. 5a) and the patients with low and high BRCA1 scores (median RFS 14.2 vs. 24.3 months, $P = 0.0065$, Fig. 5b) by univariate analysis using log-rank test. The patients with CK14 positive (median RFS 19.6 vs. 20.9 months, $P = 0.038$) also showed worse outcome than those with CK14 negative.

A low BRCA1 score and EGFR positivity were identified as independent risk factors for relapse in Cox-regression multivariate analysis (HR, 2.18; $P = 0.012$ for BRCA1 score < 1; HR, 1.64; $P = 0.042$ for EGFR positivity; Table 4).

Univariate and multivariate analyses on OS

The median OS all 152 patients with metastases was 20.1 months (95% CI, 15.6–24.6 months). Based on univariate analysis by log-rank test, brain metastasis (8.4 vs. 23.5 months, $P < 0.0001$), hepatic metastasis (13.4 vs. 24.5 months, $P = 0.044$), a RFS < 3 years (16.7 vs. 41.3 month, $P = 0.0004$, Fig. 3b), and high histologic grade (20.1 vs. 35.4 month, $P = 0.037$) were associated with a significantly worse OS. Figure 6 shows Kaplan–Meier survival curves of metastatic OS between the patients with low and high BRCA1 scores by univariate analysis (median OS 10.3 vs. 21.5 months, $P = 0.015$ by log-rank test).

Cox-regression multivariate analysis for OS revealed a shorter RFS (within 36 months), low BRCA1 score, and a PFS ≥ 12 months was identified as independent risk factors for OS (HR, 2.632; $P = 0.029$ for RFS; HR, 3.227; $P = 0.002$ for BRCA1 score < 1; HR, 0.27; $P = 0.005$ for a PFS ≥ 12 months; Table 4).

Discussion

Although there are many reports regarding the pattern of relapse in TNBCs compared to non-TNBCs [12, 13, 17, 25–27], there is little information on prognosis according to RFS within the TNBC group.

The prevailing evidence has shown that TNBC is a heterogenous group of diseases with a different biology and clinical outcome. In this study, we evaluated the potential candidate biomarkers to explain the heterogeneity of clinical courses by RFS for TNBCs.

Table 2 Comparison of patients' characteristics according to duration of RFS ($n = 152$)

	RFS < 36 months (%) ($n = 118$)	RFS ≥ 36 months (%) ($n = 34$)	*P* value
Median age	45	45	0.864(*t* test)
Status of nodal metastasis			0.197
N0 (0)	28 (23.7%)	8 (23.5%)	
N1 (1–3)	49 (41.5%)	13 (38.2%)	
N2 (4–9)	18 (15.3%)	10 (29.4%)	
N3 (≥10)	23 (19.5%)	3 (8.8%)	
Stage			
1	20 (17.4%)	5 (15.2%)	0.109
2	47 (40.9)	20 (60.6)	
3	48 (41.7)	8 (24.2)	
Histologic type			
IDC	107 (90.7%)	30 (88.3%)	0.170
Mucinous	1 (2.9%)	2 (5.9%)	
Invasive micropapillary	3 (2.5%)	0	
Poorly differentiated	2 (1.7%)	0	
Metaplastic	3 (2.5%)	0	
Adenoid cystic	1 (0.8%)	0	
Medullary	1 (0.8%)	1 (2.9%)	
Unknown	0 (0.8%)	1 (2.9%)	
Histologic grade ($n = 115$)			0.389
Grade 1	2 (2.1%)	0	
Grade 2	20 (20.6%)	6 (33.3%)	
Grade 3	75 (77.3%)	12 (66.7%)	
Nuclear grade ($n = 121$)			0.037
Grade 1	5 (5.2%)	0	
Grade 2	25 (25.8%)	12 (50.0%)	
Grade 3	67 (69.1%)	12 (50.0%)	
Adjuvant chemotherapy	110 (94.0%)	29 (85.3%)	0.143
Adjuvant radiotherapy	80 (68.4%)	23 (67.6%)	0.936
Metastatic sites			
Lung	53 (44.9%)	17 (50.0%)	0.600
Liver	20 (16.9%)	6 (17.6%)	0.924
Brain	19 (16.1%)	1 (2.9%)	0.047
Chest wall mass	15 (25.9%)	8 (42.1%)	0.179
First-line palliative regimens chemotherapy			
Platinum plus Taxane	27 (22.9%)	4 (11.8%)	0.227
Taxane ± other agents	34 (28.8%)	8 (23.5%)	0.544
Doxorubicin based	22 (18.6%)	11 (32.4%)	0.088
Capecitabine	21 (17.8%)	5 (14.7%)	0.800
Doxorubicin plus Taxane	2 (1.7%)	3 (8.8%)	0.074
Others	4 (3.4%)	0	0.575
Response to first-line palliative CTx	41 (37.3%)	20 (64.5%)	0.007
DCR to first-line palliative CTx	60 (54.5%)	24 (77.4%)	0.022
Median OS	16.7 months	41.3 months	0.0004 (log-rank) test

RFS relapse-free survival, *AC* doxorubicin + cyclophosphamide, *DCR* disease control rate (CR, complete response + PR, partial response + SD, stable disease), *OS* overall survival, *CTx* chemotherapy

Patients with a shorter RFS had a poorer OS than patients with a longer RFS. It is not clear that whether it is due to inherent aggressiveness of the tumor or a lack of effective adjuvant treatment.

Luck et al. [14] have shown that such factors as metastasis-free interval and sites of disease in TNBC may influence survival. Our results showed that hepatic and brain metastases, and a RFS ≤ 3 years were major risk factors based on univariate analysis (data not shown). However, the significance as independent prognostic factors of the liver and brain as metastatic sites was not maintained in multivariate analysis for survival. On the contrary, our marker study using IHC revealed that BRCA1 and EGFR of these were the most valuable candidate genes to predict the clinical outcome more precisely and explain the heterogeneity of TNBC with clinical relevance. There are some reports that basal markers (EGFR, CK5/6, CK14, and CK17) might be putative biomarkers to classify TNBCs [15, 27]. Moreover, caveolin 1 and 2 expression, MAPK overexpression, and epithelial cell adhesion molecule (EpCAM) have been reported to be associated with poor prognosis in TNBCs [17, 28, 29]. This fact suggests that tumor biology may override objective higher tumor burden and have a decisive role for long-term prognosis.

Overlapping and sharing the features among TNBC, BLBC, and *BRCA1*-associated breast cancer in terms of genetic, morphologic, and biologic backgrounds ("BRCA-ness") are characteristic not only in hereditary breast cancer with *BRCA* gene mutations but also in sporadic TNBCs [30, 31]. This is the reason why DNA-damaging agents and PARP inhibitors are thought to be targeted agents for TNBCs [32, 33]. Our results are compatible with and

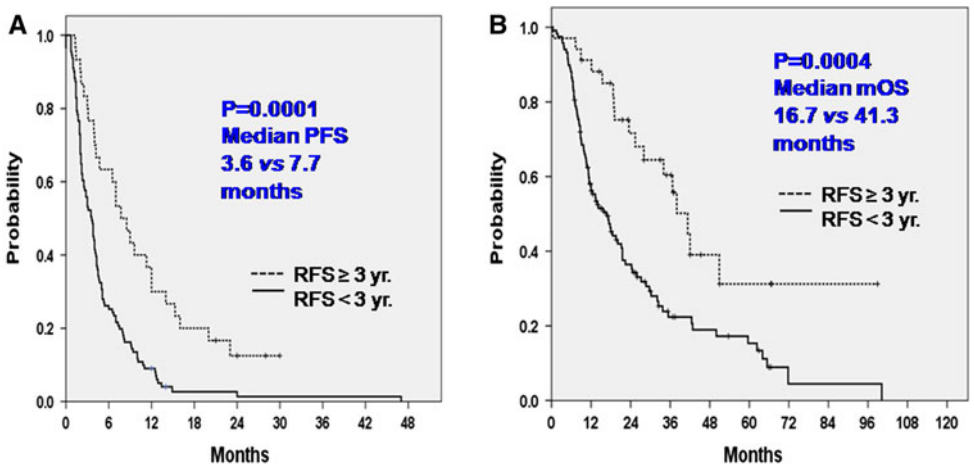

Fig. 3 PFS and metastatic OS according to RFS. **a** PFS to the first-line palliative chemotherapy according to RFS; *green line* indicates PFS of the patients whose RFS were 3 years or more; *blue line* represents PFS of the patients whose RFS were less than 3 years. **b** OS from metastasis according to RFS; *green line* represents OS whose RFS were 3 years or more; *blue line* represents OS of the patients whose RFS were less than 3 years

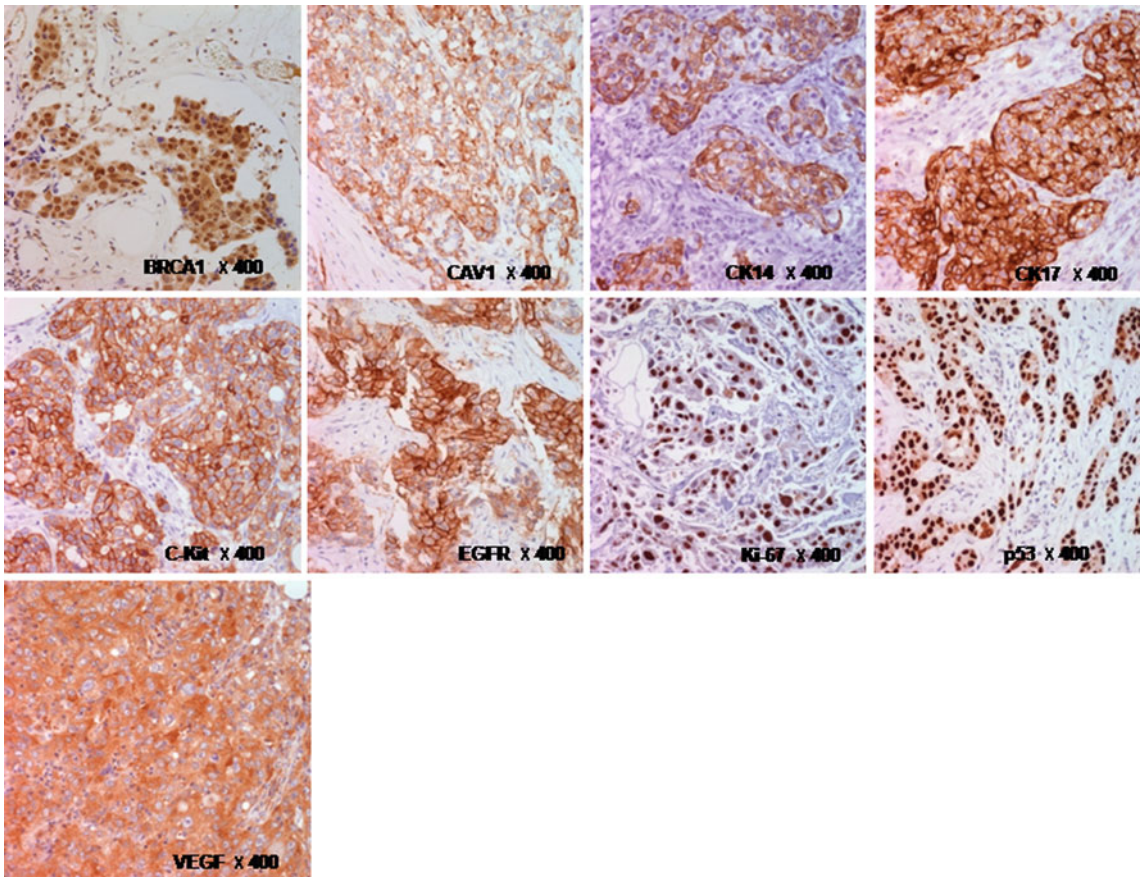

Fig. 4 Immunohistochemical staining findings

supportive of this finding. Due to "BRCAness," about 75% of TNBC seems to have defective BRCA function, even in sporadic TNBC. A low BRCA1 score in our study may represent abnormal function of the *BRCA1* gene in protein level. Consequently, it appears to be associated with shorter RFS as well as metastatic OS. Because *BRCA* gene mutation analysis cannot be performed as a routine procedure in daily clinical practice, IHC analysis for BRCA1 will probably be a useful tool to predict outcome rather than mutation analysis in TNBC. Moreover, *BRCA1* function in TNBC patients who do not have *BRCA1*-associated tumor could be impaired. In those cases, IHC might be a better method than mutation analysis. Nevertheless, it should be verified as a predictive marker for DNA-damaging agents.

Table 3 Correlation between the protein expressions by IHC and clinical outcomes according to the duration of RFS ($n = 88$)

	RFS < 36 months (%)	RFS ≥ 36 months (%)	*P* value
BRCA1 score < 1	14 (19.2%)	0	**0.064**
CAV1 score ≥ 120	3 (4.1%)	2 (13.3%)	0.200
VEGF score < 100	43 (59.7%)	13 (86.7%)	**0.041**
C-kit score ≥ 10	35 (47.9%)	5 (33.3%)	0.228
Ki67 ≥ 10%	53 (74.6%)	4 (26.7%)	**0.001**
EGFR positive	55 (76.4%)	6 (40.0%)	**0.005**
CK17 positive	42 (57.5%)	5 (33.3%)	**0.077**
CK14 positive	37 (50.7%)	1 (6.7%)	**0.001**
p53 ≥ 50%	43 (58.9%)	6 (40.0%)	0.254

Bold values indicate marginal significance

RFS relapse-free survival, *CAV1* caveolin 1, *VEGF* vascular endothelial growth factor, *EGFR* endothelial growth factor receptor

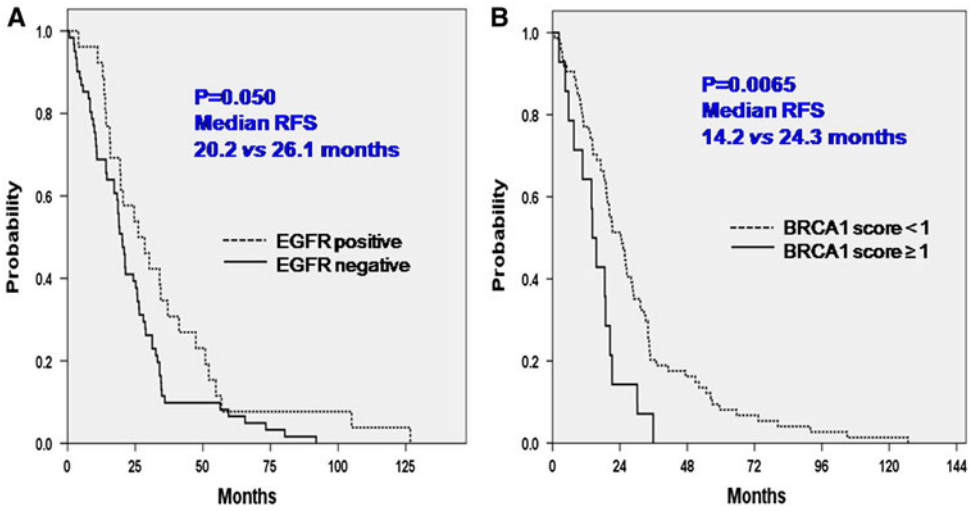

Fig. 5 RFS according to EGFR and BRCA1 stainings. **a** RFS according to EGFR staining; *dotted line* indicates RFS of the patients whose EGFR expressions were positive; *solid line* represents RFS of the patients whose EGFR expressions were negative. **b** RFS according to BRCA1 staining; *dotted line* represents RFS whose BRCA1 scores were less than 1; *solid line* represents RFS of the patients whose BRCA1 scores were 1 or more than 1

Table 4 Multivariate Cox-regression analysis including IHC on RFS and OS

		P value	Hazard ratio (HR)	95% CI	
				low	high
RFS	BRCA1 score < 1	**0.012**	2.177	1.185	3.999
	EGFR positive	**0.042**	1.638	1.017	2.639
OS	RFS < 3 year	**0.029**	**2.632**	0.928	7.461
	BRCA1 score < 1	**0.002**	**3.227**	1.565	6.653
	PFS ≥ 12 months	**0.005**	**0.274**	0.111	0.674

Bold values indicate significant *P* values

RFS relapse-free survival, *CI* confidence interval, *OS* overall survival, *PFS* progression-free survival to palliative first-line chemotherapy

EGFR is known to be a basal marker and a candidate target for TNBC [9, 14, 15]. In our analysis, EGFR expression had a positive correlation with a shorter RFS in univariate and multivariate analyses (Table 4; Fig. 5a), together with a low BRCA1 score.

Recently, there has been a report that the luminal progenitor population is a key cellular target for

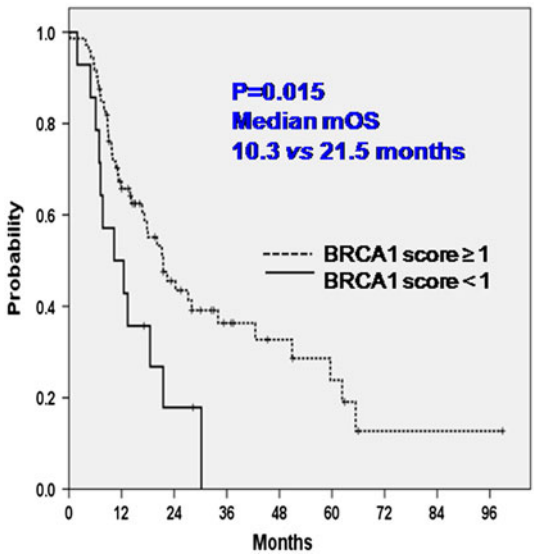

Fig. 6 Metastatic OS (mOS) according to BRCA1 staining. *Dotted line* represents mOS whose BRCA1 scores were less than 1; *solid line* represents mOS of the patients whose BRCA1 scores were 1 or more than 1

transformation in *BRCA1* pre-neoplastic tissue, and c-Kit mRNA expression is abundant in luminal progenitors [34]. They suggested molecules, such as c-Kit, may represent new targets for BLBC [14, 35]. Currently, there are many ongoing clinical trials that examine the efficacy of the new drugs, including PARP1 inhibitors, which can induce synthetic lethality in damaging BRCA1 function [36, 37]. In addition, the other targeted agents for EGFR and C-kit might have clinical utility, particularly in TNBC patients with a shorter RFS. The ongoing clinical trials for these new candidate targets [38] are expected to answer these unknown issues.

In conclusion, TNBC may be divided into at least 2 subgroups based on RFS. The patients with a RFS $\geq$ 3 years showed significantly better therapeutic outcomes in terms of DCR, PFS to palliative chemotherapy, and OS. BRCA1 and EGFR expressions were identified as independent prognostic markers to predict clinical outcomes. A prospective clinical trial with new innovative therapeutic strategies for the more aggressive subtype that has a shorter RFS with predictive biomarkers is warranted.

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Conflict of interest All authors of the paper declare no financial and personal relationships or conflict of interest.

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