Expression of p53, Ki-67, E-Cadherin, N-Cadherin and TOP2A in Triple-negative Breast Cancer

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Abstract. *Background: Elucidation of the biological features of triple negative breast cancer (TNBC) is important for deciding treatment strategies. The expression of a number of biomarkers in TNBC was analyzed to elucidate those features. Patients and Methods: The subjects were 134 TNBC patients. Immunohistochemical staining was employed to analyze for eight biomarkers: cytokeratin 5/6 (CK5/6), epidermal growth factor receptor (EGFR), p53, Ki-67 antigen (Ki-67), E-cadherin, N-cadherin, topoisomerase 2 alpha (TOP2A) and B-cell lymphoma 2 (BCL-2), which were then correlated with the nuclear grade (NG), tumor diameter, and the presence/absence of lymph node metastasis, distant recurrence and lymphatic infiltration. Results: Significantly more high than low NG TNBC exhibited positive p53, Ki-67, E-cadherin and TOP2A. High N-cadherin and TOP2A expression was shown significantly in TNBC with lymphatic infiltration, and N-cadherin was also significantly positively expressed in node metastasis-positive cases. EGFR and CK5/6 were positively expressed in high NG TNBC, but not significantly. Conclusion: Analysis for expression of p53, Ki-67, E-cadherin, N-cadherin and TOP2A is meaningful for deciding treatment strategies for TNBC.*

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DNA microarray profiling of breast cancer has resulted in classification of this malignancy into five types: luminal A, luminal B, HER2, normal-breast-like and basal-like (1). These classifications are useful in that they reflect the therapeutic efficacies of hormonal therapy, molecularly targeted drug therapies using trastuzumab, *etc*. and chemotherapy. However, classification of breast cancer by DNA microarray profiling in the clinic is difficult and today there is growing use of sub-classification based on the results of immunohistochemical (IHC) staining for estrogen receptor (ER), progesterone receptor (PgR) and HER2 (2, 3). The luminal A, luminal B and HER2 types in the classification based on ER, PgR and HER2 closely reflect their biological features, and they are practical categories for predicting the efficacy of therapeutic agents. On the other hand, breast cancer that is classified as ER-negative, PgR-negative and HER2-negative, that is, so-called triple-negative breast cancer (TNBC), does not respond well to hormonal agents or molecularly targeted drugs, leaving only chemotherapy as an effective treatment option. TNBC is currently treated with taxanes, alkylating agents, platinum agents *etc*. However, while these chemotherapeutic agents show high efficacy, various issues complicate the decision of the therapeutic strategy, such as the fact that they are followed by relatively early recurrence. Recently the classification of TNBC into basal-like and non-basal-like types on the basis of cytokeratin 5/6 (CK5/6), epidermal growth factor receptor (EGFR) staining has been recommended (4-7), and we have also reported on the importance of this approach (8). The expression profiles of biomarkers in TNBC are also

Table I. *Sources, dilution, petreatment and cutoff values of antibodies used.*

Antibody	Clone	Species	Manufacturer	Dilution	Pretreatment	Cut-off value
ER	1D5	Mouse	DakoCytomation	1:50	Boiling	≥10% (Positive)
PgR	PgR636	Mouse	DakoCytomation	1:800	Boiling	≥10% (Positive)
HER2	HER2/neu	Rabbit	DakoCytomation	Prediluted (Hercep test)	Boiling	0,1+ (Negative)
EGFR	31G7	Mouse	Nichirei Biosciences Inc.	Prediluted	Proteinase K	≥10% (Positive)
CK5/6	D5/16B4	Mouse	DakoCytomation	1:100	Autoclave	≥10% (Positive)
Ki-67	MIB-1	Mouse	DakoCytomation	1:50	Autoclave	≥20% (Positive)
p53	DO-7	Mouse	Nichirei Biosciences Inc.	Prediluted	Autoclave	≥10% (Positive)
E-Cadherin	NCH-38	Mouse	DakoCytomation	1:50	Autoclave	≥10% (Positive)
N-Cadherin	6G11	Mouse	DakoCytomation	1:50	Autoclave	≥10% (Positive)
TOP2A	Ki-S1	Mouse	DakoCytomation	1:100	Autoclave	≥10% (Positive)
BCL−2	124	Mouse	Nichirei Biosciences Inc.	Prediluted	Autoclave	≥10% (Positive)

ER: Estrogen receptor, PgR: progesterone receptor, EGFR: epidermal growth factor receptor, CK5/6: cytokeratin 5/6, Ki-67: Ki-67 antigen, TOP2A: topoisomerase II alpha, BCL-2: B-cell lymphoma 2.

gradually being elucidated. In the present study, associations between the expression of various biomarkers and the clinicopathological findings for TNBC were investigated, in the hope that the results would aid in therapeutic strategy decisions for TNBC. CK5/6 and EGFR were selected as biomarkers for determining the basal type, and p53, Ki-67, E-cadherin, N-cadherin, TOP2A and Bcl-2 as biomarkers of the degree of malignancy.

Patients and Methods

Sample collection. At Tokushima University Hospital or Tokushima Breast Care Clinic 890 patients were treated for primary breast cancer between April 2003 and March 2008. The patients with noninvasive ductal carcinoma and patients who had undergone chemotherapy prior to surgery were excluded from this study. In total, 134 patients (15%) were included in the study because they were classified as TNBC on the basis of ER (<10%), PgR (<10%) and HER2 (–) determined by IHC staining, and detailed clinicopathological studies could be performed for all of them. HER2 staining is considered to have problems with variability in the results. Accordingly, for this study, the presented HER2 results were not those generated at the time of the surgery. Rather, new sections were prepared, and IHC staining of all the patients' specimens was performed again under standardized conditions. Two pathologists evaluated the stained sections independently. HER2 was judged to be negative when the results of the Hercep test (DakoCytomation Japan, Tokyo, Japan) were 0 or +1. The clinicopathological assessment was performed in accordance with the criteria of the Japanese Breast Cancer Society (9).

Immunohistochemical staining. Paraffin-embedded tissues (4 μm thick) were used, and IHC staining was performed for CK5/6, EGFR, p53, Ki-67, E-cadherin, N-cadherin, TOP2A and BCL-2 under the conditions shown in Table I. For staining for ER and PgR, the samples were pretreated by boiling for 40 min in a TE buffer (1 mM EDTA, 10 mM Tris, pH 8.0). For staining for CK5/6, p53, Ki-67, E-cadherin, TOP2A and BCL-2, the samples were pretreated by soaking in a pH 6.0 10 mM citrate buffer and then autoclaving for 10 min. The samples were judged to be positive for CK5/6, EGFR, p53, E-cadherin, N-cadherin, TOP2A and BCL-2 when ≥10% of the cells stained positively for the respective marker. Samples were judged to be positive for Ki-67 when ≥20% of cells stained positively. $CK5/6^+$ and/or $EGFR^+$ tissues were judged to be basal-like type TNBC. The immunostained slides were independently evaluated by two of the Authors who were blinded to clinical outcome of individual patients.

Comparison of clinicopathological findings and biomarkers. The analyzed clinicopathological factors were the nuclear grade (NG 1-3) (9), tumor diameter (T1: ≤2 cm; T2: 2.1-5 cm; T3: >5 cm; T4: invasion of skin or muscle), the presence/absence of lymph node metastasis, the presence/absence of distant metastasis and the presence/absence of lymphatic infiltration. These clinicopathological factors were analyzed for association with each of the assayed biomarkers.

Statistical methods. The data were analyzed for statistical significance using the Chi-square test. A p-value of <0.05 was considered to represent a significant difference.

Results

The mean age of the 134 analyzed TNBC patients was 55±12.5 years, and 88 patients (65.6%) were ≥50 years old (Table II). A majority of the patients (73 patients; 54.4%) had a tumor diameter of T2. Nineteen (14.2%) of the patients had a tissue type other than invasive ductal carcinoma, consisting of eight patients with medullary carcinoma, three patients with invasive lobular carcinoma, three patients with squamous cell carcinoma, two patients with mucinous carcinoma and one patient each with a matrix-producing tumor, carcinoma with cartilaginous and/or osseous metaplasia and Paget's disease of the breast. Lymph node metastasis was seen in 42 patients (31.3%), while distant metastasis was present in 29 patients (21.6%).

Table II. *Clinicopathological findings (n=134).*

Age (years)	(%)
<50	46 (34.3)
≥50	88 (65.7)
Mean	55±12.5
T	
1	51 (38.0)
2	73 (54.5)
3	7 (5.2)
4	3 (2.3)
Histology	
Invasive ductal carcinoma	115 (85.8)
Other	19 (14.2)
Nuclear grade	
1	23 (17.1)
2	32 (23.9)
3	79 (59.0)
Pathological nodal status	
Positive	42 (31.3)
Negative	92 (68.7)
Distant metastasis	
+	29 (21.6)
–	105 (78.4)

T1: ≤2 cm, T2: 2.1-5 cm, T3: >5 cm, T4: invasion of skin or muscle.

The various biomarkers showed no significant differences in expression as a function of the tumor diameter (Table III). Positive expression of N cadherin was found in significantly more patients with lymph node metastasis (71.5%) ($p<0.05$) than in lymph node-negative patients. E-Cadherin expression showed a tendency to be associated with the presence of lymph node metastasis, but the difference from node-negative cases was not statistically significant. None of the analyzed biomarkers showed a significant difference in expression as a function of the presence or absence of distant metastasis. A nuclear grade of NG3 tended to be more common in the patients with basal-like type TNBC. In addition, the NG3 patients showed significantly higher incidence of positivity for p53 ($p=0.01$), Ki-67 ($p<0.001$), E-cadherin ($p<0.05$) and TOP2A ($p<0.001$) than those with NG1 or 2. Statification for positive lymphatic infiltration gave significantly higher incidences of positivity for N-cadherin ($p<0.05$) and TOP2A ($p<0.003$). E-Cadherin positivity showed a tendency to be higher in incidence in the lymphatic-infiltration-positive stratum, but the difference did not reach statistical significance (Table III).

Discussion

In basal-like type and HER2 type breast cancer, p53 and Ki-67 show high rates of positivity, which have been extensively reported as markers of malignant grade (4, 10, 11). Together with E-cadherin and TOP2A, these were significantly more frequently positively expressed than the other markers in NG3 TNBC in the present study. When lymphatic infiltration was present, N-cadherin and TOP2A were significantly more frequently positively expressed, as was N-cadherin also when lymph node metastasis was present. None of the biomarkers showed a correlation with the tumor diameter. The fact that the degree of malignancy of tumor cells, which is represented by the NG, correlated well with the expression of biomarkers used to assess the degree of malignancy can be considered a matter of course. However, if the degree of malignancy is only assessed on the basis of the NG, the assessment might be inaccurate. In some patients the NG and the expression of Ki-67 differ. Therefore, while the expression of p53 and Ki-67 can be regarded as meaningful because it is an objective assessment of the degree of malignancy, on the other hand, the study findings indicated that lymphatic infiltration, lymph node metastasis and the tumor diameter could not be used to determine the degree of malignancy of the cancer cells.

E-Cadherin is a cancer invasion suppressor gene that encodes for a transmembrane glycoprotein having the ability to cause cell cell adhesion. E-Cadherin deficiency is characteristic of invasive lobular carcinoma. Conversely, in other types of breast cancer, E-cadherin overexpression is reported to be a predictive factor for the degree of malignancy of tissues and recurrence and also to correlate with the recurrence-free interval, and E-cadherin over-expression is thought to be useful as a prognostic factor for basal-like breast cancer (12-14). However, there is no consensus on this point since it has also been reported that E-cadherin overexpression is unrelated to the prognosis (15). N-Cadherin is present in normal fibroblasts and neurocytes, but it has been reported to be up-regulated in tumor growth and to be a factor that promotes invasiveness of tumor cells (16, 17). It has been reported that E-cadherin and N-cadherin have a heterogeneous relationship in terms of their expression, and that their expressions are switched depending on how advanced the cancer is. In this study, positive E-cadherin expression was significantly more frequent in high NG disease, whereas N-cadherin expression showed no correlation with the nuclear grade. In addition, with regard to the patient group that was positive for both lymph node metastasis and lymphatic infiltration, E cadherin expression showed a tendency to be positive, but not significantly, while the frequency of N-cadherin positivity was significantly elevated, in agreement with reports N-cadherin was also expressed in metastatic breast cancer. Thus cadherin expression may reflect the progression of malignancy as suggested previously (16, 17). BCL-2 is a regulatory factor for apoptosis, and decreased expression of BCL-2 has been reported to be a factor indicating a poor prognosis of all cancer types, including breast cancer (18, 19). The

Table III. *Expression of each biomarker (n, %) according to clinicopathological parameters.*

	Tumor diameter					Nodal status			Distant metastasis status			Nuclear grade				Lymphatic infiltration		
	T1	T2	T3	T4	*P*-value	Positive	Negative	*P*-value	Positive	Negative	*P*-value	NG1	NG2	NG3	*P*-value	Positive	Negative	*P*-value
CK5/6$^+$ and/or EGFR$^+$	23 (45)	39 (53.5)	5 (71.5)	1	0.48	19 (45.3)	49 (53.2)	0.38	12 (41.4)	56 (53.3)	0.25	7 (30.4)	21 (50)	40 (58)	0.07	16 (59.2)	25 (49)	0.38
CK5/6$^-$ and EGFR$^-$	28 (55)	34 (46.5)	2 (28.5)	2			23 (54.7)	43 (48.8)	17 (58.6)	49 (46.7)		16 (69.6)	21 (50)	29 (42)		11 (40.8)	26 (51)	
p53																		
<10%	26 (51)	34 (46.5)	0	1	0.08	19 (45.3)	42 (45.7)	0.96	13 (44.8)	48 (45.7)	0.93	15 (65.2)	23 (54.8)	23 (33.3)	0.01	11 (40.8)	20 (39.2)	0.89
≥10%	25 (49)	39 (53.5)	7	2		23 (54.7)	50 (54.3)		16 (55.1)	57 (54.3)		8 (34.8)	19 (45.2)	46 (66.7)		16 (59.2)	31 (60.8)	
Ki-67																		
<20%	22 (43.2)	31 (42.5)	4 (57)	1	0.87	21 (50)	37 (40.2)	0.28	15 (51.7)	43 (41)	0.30	20 (87)	21 (50)	17 (24.6)	<0.001	11 (40.8)	21 (41.2)	0.97
≥20%	29 (56.8)	42 (57.5)	3 (43)	2		21 (50)	55 (59.8)		14 (48.3)	62 (59)		3 (23)	21 (50)	52 (75.4)		16 (59.2)	30 (58.8)	
E-Cadherin																		
<10%	25 (49)	25 (34.3)	5 (71.5)	1	0.14	14 (33.3)	42 (45.7)	0.17	16 (55.1)	40 (38)	0.09	13 (56.5)	21 (50)	22 (31.9)	0.04	9 (33.3)	21 (41.2)	0.49
≥10%	26 (51)	48 (65.7)	2 (28.5)	2		28 (66.7)	50 (54.3)		13 (44.8)	65 (62)		10 (43.5)	21 (50)	47 (68.1)		18 (66.7)	30 (58.8)	
N-Cadherin																		
<10%	19 (37.3)	30 (41)	4 (57)	2	0.60	12 (28.5)	43 (48.8)	0.04	16 (55.1)	52 (49.5)	0.59	5 (21.7)	18 (42.9)	32 (46.3)	0.11	6 (22.2)	24 (47)	0.03
≥10%	32 (62.7)	43 (59)	3 (43)	1		30 (71.5)	49 (53.2)		13 (44.8)	53 (50.5)		18 (78.3)	24 (57.1)	37 (53.6)		21 (77.8)	27 (53)	
TOP2A																		
<10%	26 (51)	40 (54.7)	1 (14.3)	1	0.20	20 (47.6)	48 (52.2)	0.62	24 (82.8)	90 (85.7)	0.69	19 (82.6)	23 (54.8)	26 (37.7)	0.0007	7 (25.9)	31 (60.8)	0.003
≥10%	25 (49)	33 (45.3)	6 (85.7)	2		22 (52.4)	44 (47.8)		5 (17.2)	15 (14.3)		4 (17.4)	19 (45.2)	43 (62.3)		20 (74.1)	20 (39.2)	
BCL-2																		
<10%	42 (82.3)	63 (86.3)	6 (85.7)	3	0.82	37 (88)	77 (83.7)	0.50	10 (34.5)	45 (42.9)	0.51	20 (87)	38 (90.5)	56 (81.2)	0.39	23 (85.2)	45 (88.2)	0.70
≥10%	9 (17.6)	10 (13.7)	1 (14.3)	0		5 (12)	15 (16.3)		19 (65.5)	60 (57.1)		3 (23)	4 (9.5)	13 (18.8)		4 (14.8)	6 (11.8)	

published literature indicates that the rate of BCL-2 positivity is high in luminal A, luminal B and HER2 type breast cancer, whereas expression of BCL-2 is low in TNBC, as found in this study and no significant differences in BCL-2 expression were shown between any of the analyzed clinicopathological parameters. TOP2A is involved in DNA synthesis and is a target molecule of anthracyclines. TOP2A expression was significantly more frequently positive in the TNBC patients with a high NG and lymphatic infiltration. Taking into consideration that the *TOP2A* gene and the *HER2* gene are thought to be located close to each other on chromosome 17, the finding that TOP2A was positive in TNBC might be perceived as odd. However, since anthracyclines are effective in many TNBC patients, it may be a reasonable finding. Further investigation along this line is warranted (20, 21).

In this study, we did not find any associations between the presence or absence of distant metastasis and any of the analyzed biomarkers. It can be thought that one reason for this was lack of uniformity of the adjuvant therapies between the two participating institutions. Accordingly, no investigation was made with regard to the outcome. Additionally, the analyzed patient population in this study was small. Further studies are proposed using a larger number of patients and a standardized therapeutic approach and analyzing for relationships of the biomarkers to the disease prognosis and outcome.

Conclusion

Analysis for expression of p53, Ki-67, E-cadherin, N-cadherin and TOP2A is meaningful for deciding the treatment strategy for TNBC.

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