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Clinicopathologic features of molecular subtypes of triple negative breast cancer based on immunohistochemical markers

Junjeong Choi[1], Woo-Hee Jung[2] and Ja Seung Koo[2]

[1]Department of Pathology, Yonsei University Wonju College of Medicine, Wonju, South Korea and [2]Yonsei University College of Medicine, Seoul, South Korea

Summary. This study was performed to identify molecular subtypes of triple negative breast carcinoma (TNBC) based on immunohistochemical markers. We prepared a tissue microarray from TNBC specimens of 122 patients and performed immunohistochemical staining for cytokeratin (CK) 5/6, epidermal growth factor receptor (EGFR), claudin 3, claudin 4, claudin 7, E-cadherin, androgen receptor (AR), and gammma-glutamyltransferase (GGT1). Based on immuno-reactivity, tumors were classified into basal-like (CK5/6 positive and/or EGFR positive), molecular apocrine (AR positive and/or GGT1 positive), claudin low (claudin 3, claudin 4, claudin 7 negative and/or E-cadherin negative), mixed (tumors belonging to two or more subtypes), and null (tumors not matching any other subtypes). The TNBC specimens of 122 patients included 27 basal-like (22.1%), 28 claudin low (23.0%), 12 molecular apocrine (9.8%), 23 mixed (18.9%) and 32 null (26.2%) subtype tumors. The molecular apocrine subtype showed the highest percentage of apocrine differentiation and the lowest Ki-67 labeling index ($p<0.001$ and $p=0.040$, respectively). In univariate analysis, tumor cell discohesiveness was related with shorter disease free survival (DFS) and overall survival (OS) ($p=0.005$, and 0.002, respectively). In multivariate analysis, tumor cell discohesiveness was related with shorter OS and CK5/6 positivity ($p=0.018$), and claudin 7 positivity ($p=0.019$) was related with shorter DFS. In conclusion, using immunohistochemical staining for CK5/6, EGFR, claudin 3, claudin 4, claudin 7, E-cadherin, AR, and GGT1, we categorized TNBC into a basal-like subtype, a claudin low subtype, a molecular apocrine subtype, a mixed subtype showing characteristics of two different subtypes, and a null subtype not belonging to any of the subtypes identified.

Key words: Breast cancer, Immunohistochemistry, Triple negative

Offprint requests to: Dr. J.S. Koo, Department of Pathology, Yonsei University College of Medicine, Severance Hospital, 50 Yonsei-ro, Seodaemun-gu, Seoul 120-752, Korea. e-mail: kjs1976@yuhs.ac

Introduction

The historical classification of breast cancer based on histological features may now be extended with molecular biological techniques, which reveal at least five clinically important subtypes based on gene expression profiles. These include luminal A, luminal B, HER-2, normal breast-like, and basal-like subtypes (Perou et al., 2000, Sorlie et al., 2001). In addition to gene expression patterns, distinctive clinical features may characterize these subtypes.

Triple negative breast cancer (TNBC), representing 10-17% of all breast cancers, is defined by the absence of estrogen receptor (ER), progesterone receptor (PR) and HER-2 expression (Haffty et al., 2006; Harris et al., 2006; Bauer et al., 2007; Carey et al., 2007; Dent et al., 2007; Tischkowitz et al., 2007; Rakha et al., 2009). Based on gene expression profiles in breast cancer patients, TNBC may be stratified into basal-like (39-54%), claudin-low (25-39%), HER-2 enriched/molecular apocrine (7-14%), luminal B (4-7%), luminal A (4-5%), and normal breast-like (1%) subtypes (van de Vijver et al., 2002; van 't Veer et al., 2002; Hess et al., 2006; Prat et al., 2010). Thus, TNBC represents a heterogeneous group of tumors that may differ in clinicopathologic features and, accordingly, in therapeutic requirements. Even though several studies tried to classify TNBC according to surrogate immunohistochemistry (IHC) markers, especially in the basal-like type, the standardized criteria for IHC interpretation has not been

made (Nielsen et al., 2004; Cheang et al., 2008; Badve et al., 2011). As for the claudin low type and molecular apocrine type, there is a limited number of reported studies on the associated surrogate IHC markers.

The aim of this study was to classify TNBC into molecular subtypes based on immunohistochemical markers and to compare clinicopathologic characteristics of the subtypes.

Materials and methods

Patient selection and clinicopathologic analysis

Patients diagnosed with triple negative breast cancer during the period from January 2000 to December 2005 were included. All patients were shown to have invasive ductal carcinoma, not otherwise specified (NOS) by pathologists. All patients had postoperative standard adjuvant therapy (chemotherapy or radiation therapy) based on their tumor stages. TNBC was defined by negative immunohistochemical testing for ER, PR, and HER-2 expression and also by negative testing for HER-2 amplification by fluorescence in situ hybridization (FISH). The ER and PR immunohistochemistry was considered positive when more than 1% of invasive tumor cells showed receptor expression (Hammond et al., 2010). HER-2 staining was scored according to the American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guideline using the following categories: 0, no immunostaining; 1+, weak incomplete membranous staining in any proportion of tumor cells; 2+, complete membranous staining, either nonuniform or weak in at least 10% of tumor cells; and 3+, uniform intense membranous staining in >30% of tumor cells (Wolff et al., 2007). Specimens with 0 to 1+ were regarded as negative and those with 3+ were considered positive. An HER-2 2+ was tested further with FISH (Vysis PathVysion HER-2 kit) for HER-2 gene amplification. As proposed by the ASCO/CAP guideline, an absolute HER-2 gene copy number lower than 4 or HER-2 gene/chromosome 17 copy number ratio (HER-2/Chr17 ratio) of less than 1.8 was considered HER-2 negative; an absolute HER-2 copy number between 4 and 6 or HER-2/Chr17 ratio between 1.8 and 2.2 was considered HER-2 equivocal; and an absolute HER-2 copy number greater than 6 or HER-2/Chr17 ratio higher than 2.2 was considered HER-2 positive (Wolff et al., 2007).

Formalin-fixed and paraffin-embedded tissue specimens from 122 cases of primary breast cancer were included. All archival hematoxylin and eosin (H&E)-stained slides for each case were reviewed by two pathologists (Koo JS, and Jung W). Histological grade was assessed using the Nottingham grading system (Elston and Ellis, 1991). Tumor staging was based on the 6th American Joint Committee on Cancer (AJCC) criteria. Disease-free survival (DFS) was measured from the date of the first curative surgery to the date of the first loco-regional or systemic relapse or death without any type of relapse. Overall survival (OS) was calculated from the date of the first curative operation to the date of the last follow-up or death from any cause. Histological parameters were evaluated from H&E-stained slides. We investigated specimens for the presence of the following pathological features: tumor margin (infiltrative or expanding), central acellular zone, central necrotic zone, central fibrotic zone, lymphocytic infiltration, tumor cell discohesiveness, and apocrine differentiation. Tumor discohesiveness in this study was defined when at least 50% of tumor cell population showed loss of cell to cell cohesiveness. Apocrine differentiation was defined by abundant granular eosinophilic cytoplasm, cytoplasmic vacuolization, and vesicular nuclei with prominent nucleoli in more than 10% of tumor cells. Clinical parameters evaluated for each tumor included patient age at initial diagnosis, lymph node status, local recurrence, systemic recurrence, and patient survival.

Tissue microarray

On H&E-stained slides of tumor tissue, a representative area was selected and a corresponding spot was marked on the surface of the paraffin block. Using a punch machine, the selected area was punched out and a 3-mm tissue core was placed into a 6 x 5 recipient block. More than two tissue cores were extracted to minimize extraction bias. Each tissue core was assigned a unique tissue microarray location number that was linked to a database containing other clinicopathologic data. The Institutional Review Board of Severance Hospital approved this study.

Immunohistochemistry (IHC)

The antibodies used for immunohistochemistry in this study are shown in Table 1. Formalin-fixed, paraffin-embedded tissue sections from tissue microarray were used for IHC. The 5 μm sections were deparaffinized in xylene and rehydrated through a graded alcohol series to distilled water. The slides were

Table 1. Clone, dilution, and source of antibodies used.

Antibody	Clone	Dilution	Company
Basal-like related			
Cytokeratin 5/6	D5/16B4	1:50	DAKO, Glostrup, Denmark
EGFR	EGFR.25	1:50	Novocastra, Newcastle, UK
Claudin-low related			
Claudin 3	Polyclonal	1:50	Abcam, Cambridge, UK
Claudin 4	Polyclonal	1:100	Abcam, Cambridge, UK
Claudin 7	Polyclonal	1:100	Abcam, Cambridge, UK
E-cadherin	36B5	1:100	Novocastra, Newcastle, UK
Molecular apocrine related			
Androgen receptor	AR441	1:50	DAKO, Glostrup, Denmark
GGT1	IgG2A	1:50	Abcam, Cambridge, UK
Proliferation related			
Ki-67	MIB-1	1:150	DAKO, Glostrup, Denmark

EGFR, epidermal growth factor receptor.

subjected to antigen retrieval by microwave irradiation, and primary antibodies were then applied.

After incubation with primary antibodies, binding was detected with biotinylated anti-mouse immunoglobulin, followed by peroxidase-labeled streptavidin with 3,3'-diaminobenzidine chromogen as a substrate. Optimal primary antibody incubation time and concentration were determined by serial dilution for each immunohistochemical assay using a tissue block fixed and embedded. Slides were counterstained with Harris hematoxylin. Two pathologists (Koo S.K. and Jung W.) interpreted the staining using a multiview microscope.

Interpretation of immunohistochemical staining

All immunohistochemical markers were assessed by light microscopy. Slides were scored according to the percentage of tumor cells exhibiting nuclear [androgen receptor (AR)], cytoplasmic [GGT1, cytokeratin (CK) 5/6], and membranous (EGFR, claudin-3, claudin-4, claudin-7, and E-cadherin) staining. Results for AR, CK5/6, and GGT1 were considered positive when more than 10% of the tumor cells were stained. Results for EGFR, claudin 3, claudin 4, claudin 7, and E-cadherin were classified as negative, weak, moderate, or intense expression. Moderate or intense staining was considered positive. Results for Ki-67 were scored by counting the positively stained nuclei and expressing this number as a percentage of the total tumor cell number [Ki-67 labeling index (LI)].

Molecular classification of TNBC according to IHC

Based on IHC results, TNBC was classified into *basal-like* (CK5/6 positive and/or EGFR positive), *molecular apocrine* (AR positive and/or GGT1 positive), *claudin low* (claudin 3, claudin 4, claudin 7 negative and/or E-cadherin negative), *mixed* (showing characteristics of 2 different subtypes), and *null* (tumors not belong to any types described) subtypes (Figures 1 and 2).

Statistical analysis

Data were analyzed using SPSS for Windows, Version 12.0 (SPSS Inc., Chicago, IL, USA). Student's t-test and Fisher's exact test were used for continuous and categorical variables, respectively. Kaplan-Meier analysis and log-rank statistics were used to evaluate time to tumor recurrence and overall survival. Multivariate regression analysis was performed using the Cox proportional hazards model. Statistical significance was accepted for $p<0.05$.

Results

Clinicopathologic characteristics of TNBC

The clinicopathologic characteristics of 122 patients with TNBC are summarized in Table 2. A central acellular zone was present in 34 tumors (27.9%) and dense lymphocytic infiltration in 30 tumors (24.6%). Tumor discohesiveness, indicated by a tendency of tumor cells to separate, was noted in 10 tumors (8.2%) and an expanding tumor margin was noted in 98 (80.3%). Apocrine differentiation was observed in 19 tumors (15.6%).

Immunohistochemical results and sublcassifications of TNBC

The results of IHC are shown in Table 3. Based on these results, the 122 cases of TNBC were classified into

Table 2. Clinicopathologic characteristics of TNBC.

Parameter	Number of patient (N=122) (%)
Age (years, mean ± SD)	47.5±12.1
Histologic grade	
I	4 (3.3)
II	41 (33.6)
III	77 (63.1)
Tumor stage	
T1	40 (32.8)
T2	78 (63.9)
T3	4 (3.3)
Nodal stage	
N0	76 (62.3)
N1	31 (25.4)
N2	9 (7.4)
N3	6 (4.9)
Central acellular zone	
Yes	34 (27.9)
No	88 (72.1)
Central necrotic zone	
Yes	10 (8.2)
No	112 (91.8)
Central fibrotic zone	
Yes	27 (22.1)
No	95 (77.9)
Lymphocytic infiltration	
No	37 (30.3)
Mild	55 (45.1)
Marked	30 (24.6)
Tumor cell discohesiveness	
Yes	10 (8.2)
No	112 (91.8)
Tumor margin	
Expanding	98 (80.3)
Infiltrative	24 (19.7)
Apocrine differentiation	
Present	19 (15.6)
Absent	103 (84.4)
Tumor cell necrosis (%, mean ± SD)	11.3±15.9
Tumor recurrence	19 (15.6)
Patient death	16 (13.1)
Duration of clinical follow-up (months, mean ± SD)	59.5±27.0

TNBC, triple negative breast carcinoma.

subtypes, including 27 basal-like (22.1%), 28 claudin low (23.0%), 12 molecular apocrine (9.8%), 23 mixed (18.9%), and 32 null (26.2%) subtype tumors. The 23 mixed TNBCs included 12 basal-like + claudin low, 5 basal like + molecular apocrine, and 6 claudin low + molecular apocrine tumors.

Clinicopathologic characteristics according to the subtype of TNBC

The clinicopathologic parameters according to TNBC subtypes are shown in Table 4. The patients with molecular apocrine tumors were the oldest in the group (p=0.049). Histological grade III was most common in all subtypes except for the claudin low type and molecular apocrine type. The rate of lymph node metastasis was highest in the molecular apocrine type, although not statistically significant (p=0.681).

Histological features of the central acellular zone and central fibrotic zone were found in the basal-like TNBCs most frequently, although this was not statistically significant (p=0.116, and p=0.135, respectively). All subtypes showed tumor cell discohesiveness except for the null subtype, and an infiltrative tumor margin was more frequently observed in the claudin-low and molecular apocrine subtypes (p=0.063). Apocrine differentiation was most frequently observed in the molecular apocrine TNBC (p<0.001), which also showed the lowest Ki-67 labeling index of all the subtypes (p=0.004).

Clinicopathologic features among mixed type TNBC

Table 5 shows the analysis for 23 cases of TNBCs with mixed subtypes. Basal like and molecular apocrine tumors showed a higher histological grade than the other two subtypes (p=0.048) and lymph node metastasis occurred more frequently in the claudin low and molecular apocrine subtype. Histological features of central acellular zone, central necrotic zone, and central fibrotic zone were observed only in the basal like & claudin low subtype, which, in contrast to the other two subtypes, displayed no apocrine differentiation. The claudin low & molecular apocrine TNBC showed a trend toward the lowest proportion of tumor cell necrosis among the subtypes (p=0.150).

The impact of pathologic parameters and immunohistochemical results on tumor recurrence, patient survival, disease-free survival, and overall survival

The correlations between histopathological parameters and immunohistochemical subtypes with tumor recurrence and patient survival are summarized in Table 6. In univariate analysis, the histological finding of tumor cell discohesiveness was significantly correlated with patient survival. (p=0.010). Tumors with cell discohesiveness (p=0.005), CK5/6 positivity (p=0.050), and claudin 7 positivity (p=0.034) and tumors with no lymphocytic infiltration (p=0.034) were associated with shorter DFS. Tumor cell discohesiveness was associated with shorter OS (p=0.002). Multivariate regression analysis with the Cox proportional hazard model showed positive correlations of lymph node metastasis (p<0.001, hazard ratio: 8.519), young age (≤35 years, p=0.045, hazard ratio: 3.050), CK5/6 positivity (p=0.018, hazard ratio: 3.097), and claudin 7 positivity (p=0.019, hazard ratio: 15.624) with shorter DFS and lymph node metastasis (p=0.008, hazard ratio: 4.837) and tumor cell discohesiveness (p=0.048, hazard ratio: 3.262) with shorter OS (Table 7).

DFS and OS according to TNBC subtypes are shown in Figure 3. The basal-like and null subtypes showed less favorable prognosis, whereas the molecular apocrine subtype showed better prognosis than others. The claudin low and mixed subtypes showed intermediate prognosis. However, these differences were not statistically significant.

Table 3. Immunohistochemical results and subclassification of TNBC.

Antibody	Number of patient (N=122) (%)
Cytokeratin 5/6	
Positive	33 (27.0)
Negative	89 (73.0)
EGFR	
Positive	16 (13.1)
Negative	106 (86.9)
Claudin 3	
Positive	45 (36.9)
Negative	77 (63.1)
Claudin 4	
Positive	59 (48.4)
Negative	63 (51.6)
Claudin 7	
Positive	2 (1.6)
Negative	120 (98.4)
E-cadherin	
Positive	106 (86.9)
Negative	16 (13.1)
Androgen receptor	
Positive	7 (5.7)
Negative	115 (94.3)
GGT-1	
Positive	23 (18.9)
Negative	99 (81.1)
Immunophenotype	
Basal-like type	27 (22.1)
Claudin low type	28 (23.0)
Molecular apocrine type	12 (9.8)
Null type	32 (26.2)
Mixed type	23 (18.9)
Basal like + claudin low type	12 (52.2)
Basal like + molecular apocrine type	5 (21.7)
Claudin low + molecular apocrine type	6 (26.1)

EGFR, epidermal growth factor receptor; TNBC, triple negative breast carcinoma.

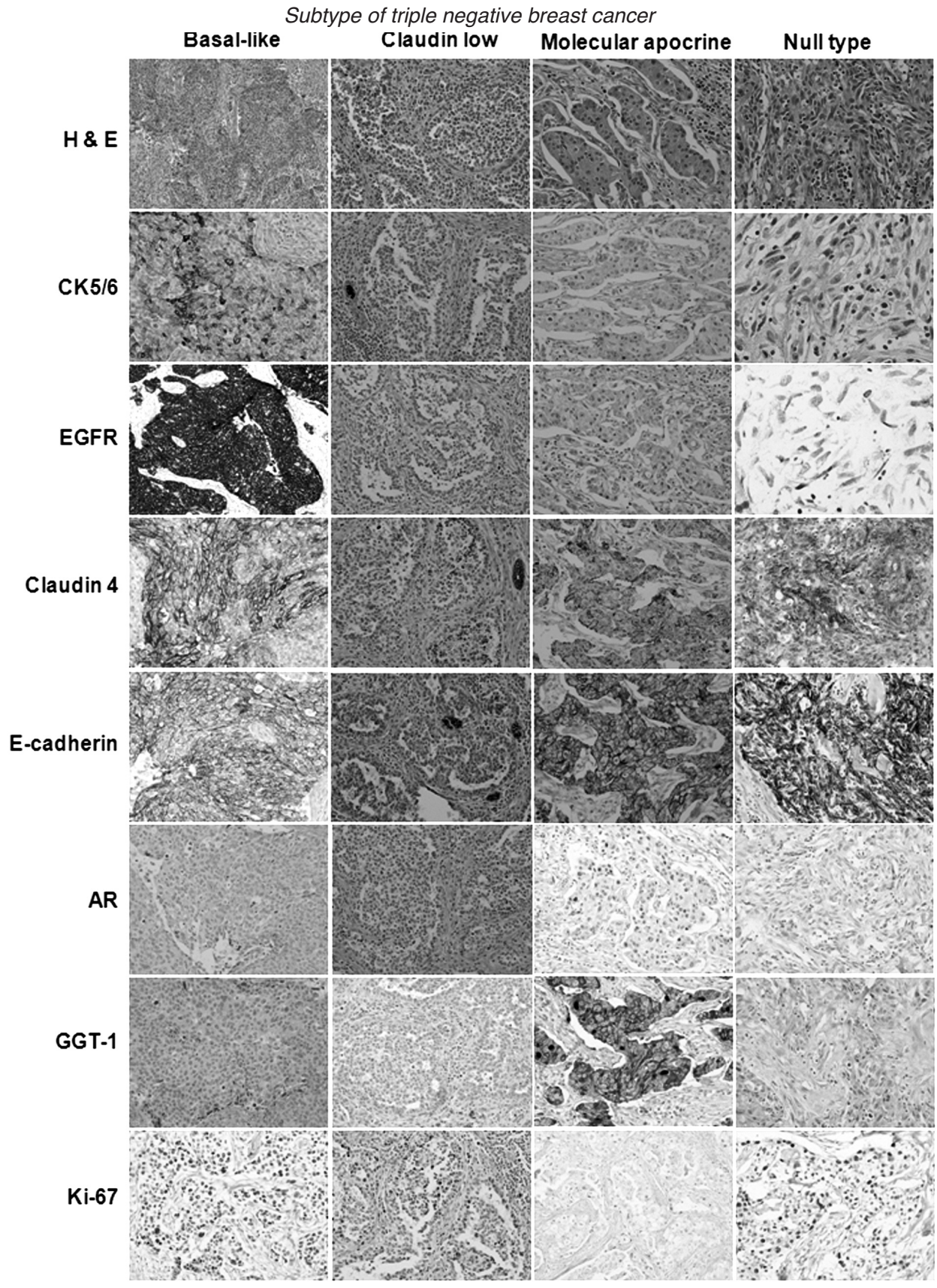

Fig. 1. Histologic and immunohistochemical features defining subtypes of triple negative breast cancer. Basal-like TNBC expressed cytokeratin 5/6, epidermal growth factor receptor (EGFR), claudin 4, and E-cadherin. Claudin low TNBC showed negative reactivity to claudin 4 and E-cadherin. Molecular apocrine TNBC expressed claudin 4, E-cadherin, androgen receptor, and GGT1. Null subtype TNBC expressed only claudin 4 and E-cadherin. x 200

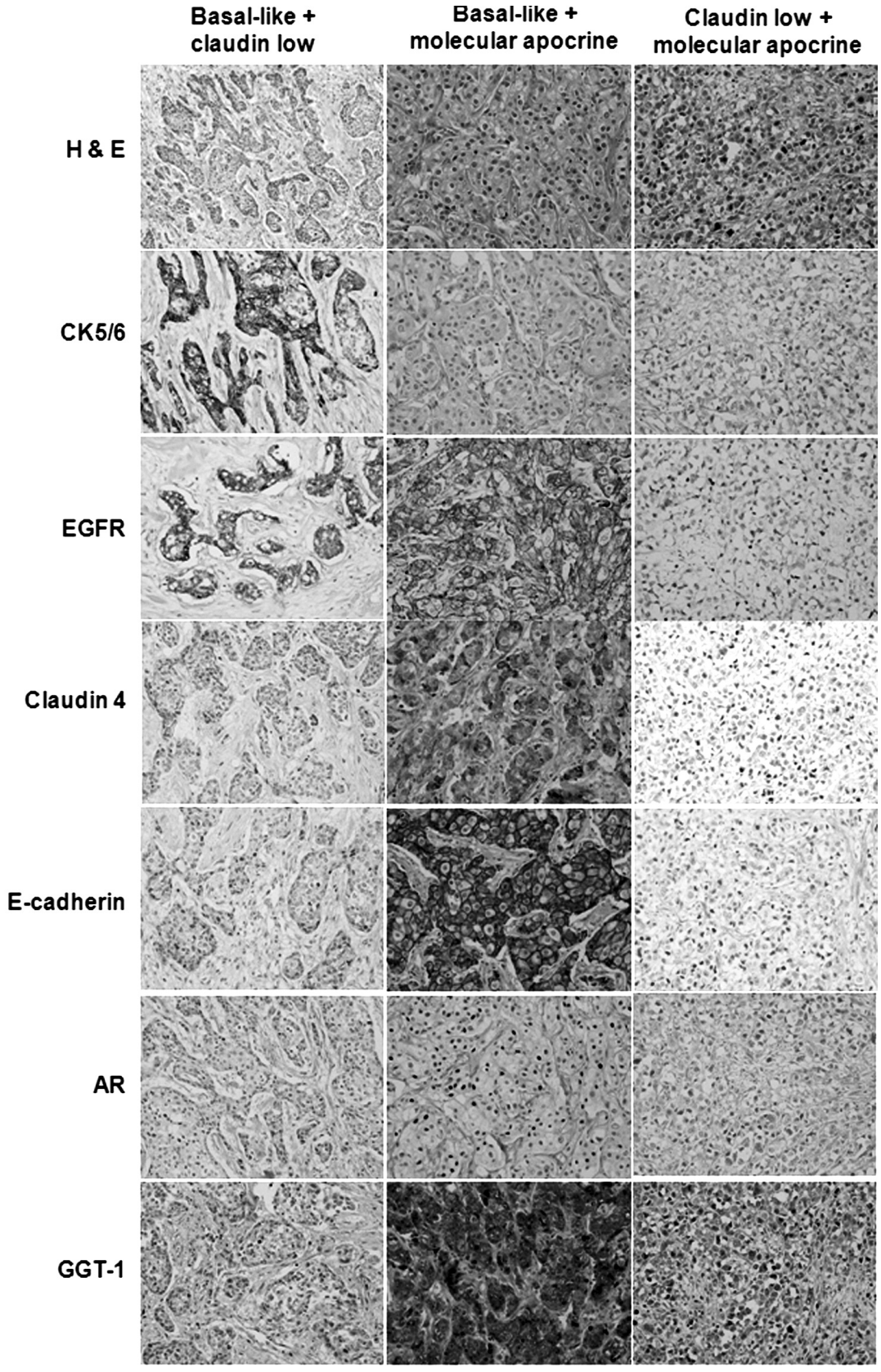

Fig. 2. Histologic and immunohistochemical features of triple negative breast cancer of mixed subtype. Basal-like plus claudin low subtype TNBC showed immunoreactivity to cytokeratin 5/6 and epidermal growth factor receptor (EGFR), but not to claudin 4 or E-cadherin. Basal-like plus molecular apocrine TNBC expressed EGFR, androgen receptor, and GGT1. Claudin low plus molecular apocrine TNBC showed immunoreactivity to GGT1 but not to claudin 4 or E-cadherin. x 200

Discussion

Based on immunohistochemical profiles, we classified 122 cases of TNBC into 5 subtypes, including basal-like (22.1%), claudin low (23.0%), molecular apocrine (9.8%), null (26.2%), and mixed (18.9%) subtypes. We also investigated clinicopathologic characteristics according to the subtypes. These findings are consistent with the heterogeneous behavior of TNBC.

In this study set, the null subtype showed the highest frequency, in contrast to previous reports that showed the basal-like subtype to be predominant in TNBC (Perou et al., 2000; van 't Veer et al., 2002; Cheang et al.,

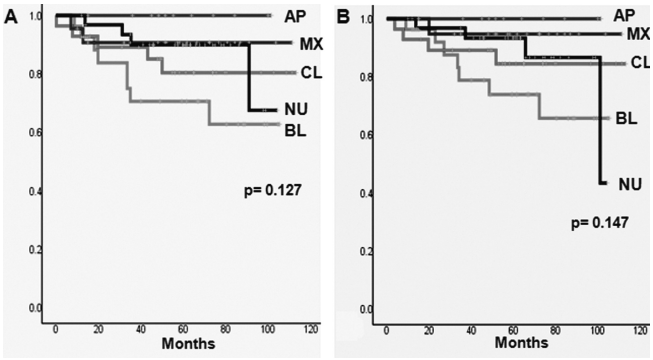

Fig. 3. Disease-free survival **(A)** and overall survival curves **(B)** for the following subtypes of triple negative breast cancer: BL, basal-like; NU, null; CL, claudin low; MX, mixed; AP, molecular apocrine.

Table 4. Clinicopathologic features according to subtype of TNBC

Parameter	Total (n=122) (%)	Basal-like type (n=27) (%)	Claudin low type (n=28) (%)	Molecular apocrine type (n=12) (%)	Null type (n=32) (%)	Mixed type (n=23) (%)	P-value
Age (years, mean ± SD)	47.5±12.1	47.0±12.3	47.8±11.8	56.9±13.7	44.4±11.6	47.1±10.2	0.049
Histologic grade							0.109
I	4 (3.3)	2 (7.4)	1 (3.6)		1 (3.1)		
II	41 (33.6)	7 (25.9)	14 (50.0)	7 (58.3)	6 (18.8)	7 (30.4)	
III	77 (63.1)	18 (66.7)	13 (46.4)	5 (41.7)	25 (78.1)	16 (69.6)	
Tumor stage							0.791
T1	40 (32.8)	8 (29.6)	11 (39.3)	5 (41.7)	8 (25.0)	8 (34.8)	
T2	78 (63.9)	18 (66.7)	16 (57.1)	6 (50.0)	24 (75.0)	14 (60.9)	
T3	4 (3.3)	1 (3.7)	1 (3.6)	1 (8.3)		1 (4.3)	
Nodal stage							0.681
N0	76 (62.3)	16 (59.3)	19 (67.9)	6 (50.0)	20 (62.5)	15 (65.2)	
N1	31 (25.4)	5 (18.5)	6 (21.4)	6 (50.0)	9 (28.1)	5 (21.7)	
N2	9 (7.4)	4 (14.8)	1 (3.6)		2 (6.3)	2 (8.7)	
N3	6 (4.9)	2 (7.4)	2 (7.1)		1 (3.1)	1 (4.3)	
Central acellular zone							0.116
Yes	34 (27.9)	13 (48.1)	6 (21.4)	2 (16.7)	8 (25.0)	5 (21.7)	
No	88 (72.1)	14 (51.9)	22 (78.6)	10 (83.3)	24 (75.0)	18 (78.3)	
Central necrotic zone							0.563
Yes	10 (8.2)	3 (11.1)	1 (3.6)		4 (12.5)	2 (8.7)	
No	112 (91.8)	24 (88.9)	27 (96.4)	12 (100.0)	28 (87.5)	21 (91.3)	
Central fibrotic zone							0.135
Yes	27 (22.1)	11 (40.7)	5 (17.9)	2 (16.7)	5 (15.6)	4 (17.4)	
No	95 (77.9)	16 (59.3)	23 (82.1)	10 (83.3)	27 (84.4)	19 (82.6)	
Lymphocytic infiltration							0.567
No	37 (30.3)	7 (25.9)	9 (32.2)	5 (41.7)	9 (28.1)	7 (30.4)	
Mild	55 (45.1)	14 (51.9)	16 (57.1)	4 (33.3)	12 (37.5)	9 (39.2)	
Marked	30 (24.6)	6 (22.2)	3 (10.7)	3 (25.0)	11 (34.4)	7 (30.4)	
Tumor cell discohesiveness							0.180
Yes	10 (8.2)	4 (14.8)	4 (14.3)	1 (8.3)		1 (4.3)	
No	112 (91.8)	23 (85.2)	24 (85.7)	11 (91.7)	32 (100.0)	22 (95.7)	
Tumor margin							0.063
Expanding	98 (80.3)	24 (88.9)	18 (64.3)	8 (66.7)	28 (87.5)	20 (87.0)	
Infiltrative	24 (19.7)	3 (11.1)	10 (35.7)	4 (33.3)	4 (12.5)	3 (13.0)	
Apocrine differentiation							< 0.001
Present	19 (15.6)	3 (11.1)	1 (3.6)	8 (66.7)	4 (12.5)	3 (13.0)	
Absent	103 (84.4)	24 (88.9)	27 (96.4)	4 (33.3)	28 (87.5)	20 (87.0)	
Tumor cell necrosis (%, mean ± SD)	11.3±15.9	9.2±12.3	7.8±13.0	7.5±14.2	13.4±16.3	16.9±21.4	0.206
Ki-67 LI (%, mean ± SD)	21.5±22.5	27.7±23.5	17.6±17.8	5.5±5.2	25.3±26.1	22.1±23.5	0.004

LI, labeling index; TNBC, triple negative breast carcinoma.

2008; Rakha et al., 2009; Badve et al., 2011). The discrepancy may be attributed to the lack of a unified definition of basal-like carcinoma. Basal-like breast cancer (BLBC) was initially defined by microarray-analysis (gene profiling), but immunohistochemical markers have been proposed to provide a practical alternative for clinical use. Unfortunately, the immunohistochemical criteria used to define BLBC are not standardized (Nielsen et al., 2004; Cheang et al., 2008; Badve et al., 2011). These criteria include the following: (1) lack of ER, PR, and HER-2 expression ('triple-negative' immunophenotype); (2) expression of one or more high-molecular-weight basal cytokeratins (CK5/6, CK14, and CK17); (3) lack of ER and HER-2 expression in conjunction with expression of CK5/6 and/or epidermal growth factor receptor (EGFR); and (4) lack of ER, PR, and HER-2 expression in conjunction with CK5/6 and/or EGFR expression. We used definition

Table 5. Clinicopathologic features among mixed type TNBC.

Parameter	Total (n=23) (%)	Basal like + claudin low type (n=12) (%)	Basal like + molecular apocrine type (n=5) (%)	Claudin low + molecular apocrine type (n=6) (%)	P-value
Age (years, mean ± SD)	47 1±10.2	45.3±9.3	52.8±14.3	46.1±10.2	0.399
Histologic grade					0.048
I					
II	7 (30.4)	3 (25.0)		4 (66.7)	
III	16 (69.6)	9 (75.0)	5 (100.0)	2 (33.3)	
Tumor stage					0.513
T1	8 (34.8)	4 (33.3)	3 (60.0)	1 (16.7)	
T2	14 (60.9)	7 (58.3)	2 (40.0)	5 (83.3)	
T3	1 (4.3)	1 (8.3)			
Nodal stage					0.161
N0	15 (65.2)	10 (83.3)	3 (60.0)	2 (33.3)	
N1	5 (21.7)	1 (8.3)	1 (20.0)	3 (50.0)	
N2	2 (8.7)	1 (8.3)		1 (16.7)	
N3	1 (4.3)		1 (20.0)		
Central acellular zone					0.053
Yes	5 (21.7)	5 (41.7)			
No	18 (78.3)	7 (58.3)	5 (100.0)	6 (100.0)	
Central necrotic zone					0.366
Yes	2 (8.7)	2 (16.7)			
No	21 (91.3)	10 (83.3)	5 (100.0)	6 (100.0)	
Central fibrotic zone					0.109
Yes	4 (17.4)	4 (33.3)			
No	19 (82.6)	8 (66.7)	5 (100.0)	6 (100.0)	
Lymphocytic infiltration					0.622
No	7 (30.4)	4 (33.3)	1 (20.0)	2 (33.3)	
Mild	9 (39.1)	5 (41.7)	3 (60.0)	1 (16.7)	
Marked	7 (30.4)	3 (25.0)	1 (20.0)	3 (50.0)	
Tumor cell discohesiveness					0.227
Yes	1 (4.3)			1 (16.7)	
No	22 (95.7)	12 (100.0)	5 (100.0)	5 (83.3)	
Tumor margin					0.619
Expanding	20 (87.0)	10 (83.3)	5 (100.0)	5 (83.3)	
Infiltrative	3 (13.0)	2 (16.7)		1 (16.7)	
Apocrine differentiation					0.079
Present	3 (13.0)		2 (40.0)	1 (16.7)	
Absent	20 (87.0)	12 (100.0)	3 (60.0)	5 (83.3)	
Tumor cell necrosis (%, mean ± SD)	16.9 ± 21.4	24.1±23.9	16.0±21.9	3.3±5.1	0.150
Ki-67 LI (%,mean ± SD)	22.1±23.5	19.1±22.3	34.4±29.3	17.8±21.5	0.438
Tumor recurrence					0.327
Yes	2 (8.7)		1 (20.0)	1 (16.7)	
No	21 (91.3)	12 (100.0)	4 (80.0)	5 (83.3)	
Disease related death					0.247
Yes	1 (4.3)			1 (16.7)	
No	22 (95.7)	12 (100.0)	5 (100.0)	5 (83.3)	

LI, labeling index; TNBC, triple negative breast carcinoma.

Table 6. The impact of pathologic parameters and immunohistochemical results on tumor recurrence, patient survival, time to tumor recurrence, and time to overall survival.

Parameter	Tumor recurrence (n=120*)			Disease free survival		Patient survival (n=120*)			Overall survival	
	Present (n=19)	Absent (n=101)	P-value	Mean survival (95% CI) months	P-value	Death (n=16)	Survival (n=104)	P-value	Mean survival (95% CI) months	P-value
Pathologic parameters										
Central acellular zone			0.370		0.487			0.382		0.460
Yes	7	27		94.4 (82.2-106.6)		6	28		97.4 (86.3- 108.5)	
No	12	74		97.1 (89.7-104.5)		10	76		99.9 (93.3-106.5)	
Central necrotic zone			0.598		0.509			0.746		0.635
Yes	1	9		96.9 (82.6-111.1)		1	9		99.0 (88.9-109.1)	
No	18	92		96.6 (89.6-103.5)		15	95		99.5 (93.2-105.9)	
Central fibrotic zone			0.103		0.111			0.123		0.110
Yes	7	20		87.3 (71.2-103.4)		6	21		90.2 (75.0-105.3)	
No	12	81		98.4 (91.6-105.2)		10	83		100.8 (94.7-106.9)	
Lymphocytic infiltration			0.038		0.034			0.124		0.107
No	10	25		79.9 (66.6-93.3)		8	27		85.7 (73.6-97.9)	
Mild	7	48		101.2 (93.0-109.4)		6	49		102.5 (94.6-110.5)	
Marked	2	28		95.8 (88.3-103.3)		2	28		96.1 (89.0-103.2)	
Tumor cell discohesiveness			0.029		0.005			0.010		0.002
Yes	4	6		61.6 (30.4-92.7)		4	6		64.9 (36.0-93.9)	
No	15	95		99.9 (93.7-106.1)		12	98		102.4 (96.7-108.0)	
Tumor margin			0.134		0.100			0.187		0.122
Expanding	13	84		99.9 (93.2-106.6)		11	86		102.0 (95.9-108.1)	
Infiltrative	6	17		85.2 (67.5-102.9)		5	18		90.1 (73.9-106.4)	
Apocrine differentiation			0.552		0.497			0.292		0.263
Present	2	16		96.3 (84.4-108.2)		1	17		100.8 (92.0-109.5)	
Absent	17	85		96.3 (89.1-103.6)		15	87		98.4 (91.6-105.2)	
Immunohistochemical parameter										
Cytokeratin 5/6			0.120		0.050			0.336		0.153
Positive	8	25		82.1 (68.2-96.1)		6	27		88.1 (75.7-100.5)	
Negative	11	76		101.0 (94.4-107.7)		10	77		102.3 (96.0-108.5)	
EGFR			0.219		0.240			0.104		0.143
Positive	4	11		82.7 (64.1-101.4)		4	11		83.4 (65.5-101.3)	
Negative	15	90		98.8 (92.2-105.5)		12	93		101.7 (95.6-107.7)	
Claudin 3			0.592		0.556			0.528		0.481
Positive	8	36		88.8 (78.4-99.2)		7	37		91.2 (81.7-100.8)	
Negative	11	65		98.7 (90.9-106.6)		9	67		101.4 (94.4-108.5)	
Claudin 4			0.683		0.553			0.886		0.705
Positive	10	48		89.8 (81.0-98.5)		8	50		92.9 (85.1-100.8)	
Negative	9	53		98.8 (90.2-107.5)		8	54		100.8 (93.0-108.7)	
Claudin 7			0.182		0.034			0.576		0.720
Positive	1	1		21.5 (1.4-41.5)		0	2		n/a	
Negative	18	100		98.0 (91.7-104.4)		16	102		n/a	
E-cadherin			0.637		0.801			0.417		0.591
Positive	16	89		98.0 (91.3-104.8)		13	92		100.8 (94.6-107.0)	
Negative	3	12		87.3 (71.7-102.9)		3	12		90.0 (74.8-105.2)	
Androgen receptor			0.237		0.255			0.285		0.314
Positive	0	7		n/a		0	7		n/a	
Negative	19	94		n/a		16	97		n/a	
GGT-1			0.297		0.393			0.159		0.214
Positive	2	21		102.0 (89.8-114.3)		1	22		106.2 (96.5-115.9)	
Negative	17	80		96.1 (88.7-103.4)		15	82		98.1 (91.3-105.0)	
Phenotype related			0.128		0.127			0.133		0.147
Basal-like	8	19		n/a		7	20		n/a	
Claudin low	5	23		n/a		4	24		n/a	
Molecular apocrine	0	12		n/a		0	12		n/a	
Null	4	27		n/a		4	27		n/a	
Mixed	2	20		n/a		1	21		n/a	

EGFR, epidermal growth factor receptor. *The data from two patients with no clinical follow-up data were not included in the analyses.

(4) for BLBC in this study, and it is possible that a significant proportion of the null-subtype TNBCs we identified would be categorized as basal-like had we used additional basal markers such as CK 14 and CK 17. Furthermore, it is possible that the luminal B, luminal A, and normal breast-like subtypes, which represent a minor proportion of TNBC, would also have been included. Further studies are needed to resolve this problem.

The basal-like subtype accounted for 22.1% of TNBCs in this study, which is lower than previously reported (39-54%) (van de Vijver et al., 2002; van 't Veer et al., 2002; Hess et al., 2006; Prat et al., 2010). If we included cases showing the basal-like immunophenotype, the basal-like subtype would account for 36.1% of all TNBCs [(27+12+5)/122)]. As noted above, this discrepancy may stem from differences between studies in defining the basal-like subtype, since neither the definition of basal markers nor the criteria for a positive immunohistochemical test have been established (Nielsen et al., 2004; Cheang et al., 2008; Badve et al., 2011). For this reason, it is suggested that the terminology "basal-like carcinoma" should not be used in the clinical pathology report (Badve et al., 2011).

The claudin low subtype accounted for 28% of TNBCs, and together with the mixed subtype, it accounted for 37.8% of TNBCs [(28+12+6)/122]. This compares favorably with a previously reported rate of 25-39%. The molecular apocrine subtype accounted for 9.8% of TNBCs, and together with the mixed subtype, it accounted for 13.9%. This also agrees with the rates previously reported (7-14%). These comparisons should be interpreted with caution, however, since the criteria for defining claudin low and molecular apocrine subtypes in the IHC protocol may have been different among these previously reported studies.

The TNBC subtypes did not show distinctive histological features, except for the molecular apocrine subtype, which showed significant apocrine differentiation. Previously suggested histological features for basal-like breast cancer, which includes high histological grade, central acellular or fibrotic zone, pushing border, and lymphocytic infiltrates (Tsuda et al., 1999, 2000; Fulford et al., 2006; Livasy et al., 2006) were also found in other subtypes. Limited differentiation, lymphocyte infiltration and metaplastic features were previously reported for the claudin low subtype (Prat et al., 2010), but we also observed these features in other subtypes in this study. Since claudin 3, 4 and 7, and E-cadherin are proteins involved with the epithelial cell tight junction and are underexpressed in claudin low subtype tumors (Herschkowitz et al., 2007), cell discohesiveness was expected in TNBC (as in the lobular carcinoma). However, all subtypes except for the null subtype also displayed discohesiveness, which did not differ significantly among the subtypes (p=0.180). Thus, the expression of claudin 3, 4, and 7 and E-cadherin as determined by IHC may not correspond directly to morphological features. Histological features of apocrine differentiation were significantly correlated with the molecular apocrine subtype as defined by IHC (p<0.001). This was consistent with previous reports (Farmer et al., 2005; Banneau et al., 2010).

As defined by molecular criteria, apocrine breast cancers are ER negative and AR positive. Since these tumors frequently display HER-2 amplification, they may share features with tumors of the HER-2-enriched subtype, including high histological grade and poor prognosis (Farmer et al., 2005; Banneau et al., 2010). In contrast to previous findings, molecular apocrine

Table 7. Multivariate analysis for survival in TNBC.

Parameter	Disease free survival			Overall survival		
	Hazard ratio	95% CI	*P*-value	Hazard ratio	95% CI	*P*-value
T stage						0.152
T1 versus T2-4	6.397	0.76-53.6	0.087	4.807	0.56-41.3	
N stage			<0.001			0.008
N0 versus N1-3	8.519	2.87-25.3		4.837	1.52-15.4	
Age						0.128
≤35 versus >35	3.050	1.02-9.08	0.045	3.078	0.72-13.1	
Histologic grade			0.262			0.250
I/II versus III	0.516	0.16-1.64		0.477	0.14-1.68	
Lymphocytic infiltration						
No	Ref.		0.124	Ref.		0.355
Mild	2.526	0.44-14.6	0.300	1.667	0.28-9.93	0.574
Marked	0.732	0.13-4.28	0.729	0.644	0.10-3.97	0.635
Tumor cell discohesiveness	0.889	0.18-4.30	0.884	3.262	1.01-10.5	0.048
CK5/6 positivity	3.097	1.21-7.91	0.018	1.390	0.18-11.0	0.755
Claudin 7 positivity	15.624	1.58-154	0.019	n/a	n/a	n/a

TNBC, triple negative breast carcinoma.

TNBCs in this study showed lower Ki-67 labeling (p=0.004), lower histological grade (p=0.109), and relatively good prognosis (p=0.147). This discrepancy in tumor aggressiveness may be related to the triple-negative status of the tumors, because the molecular apocrine tumors in this study belonged to the group of TNBCs without HER-2 overexpression and/or amplification, whereas most of those in the previously studied group did show HER-2 amplification. Further studies concerning HER-2 amplification in molecular apocrine breast cancer should be pursued.

One unique finding in this study was the presence of a mixed TNBC subtype, representing 18.9% of the study set. The gene clustering study of breast cancer did not identify a mixed type TNBC, but immunohistochemical staining did identify a TNBC subtype with characteristics of two subtypes. While detection of a mixed subtype may reflect the limited resolution of the present immunohistochemical system, the possibility exists that a single malignancy may harbor two different intrinsic phenotypes, which must be concurrently evaluated. The individual characteristics of the TNBC subtypes may be attributed to differences in origin of the tumor cells, but another possibility is that tumor cells may be arrested at different stages of epithelial cell development. In the latter case, the most primitive tumor would be the claudin low subtype followed by the basal-like and molecular apocrine/HER-2-enriched subtypes, considering that epithelial cells acquire luminal characteristics (Denkert et al., 2010) with gradual loss of basal cell characteristics (Prat and Perou, 2009). Based on this hypothesis, the development of a mixed subtype of TNBC might be explained by the arrest of tumor cells between stages. Further studies may resolve these questions.

We did not observe significant differences in survival according to TNBC subtypes, probably because the follow-up was too short and the number of cases too small; however, the molecular apocrine subtype was associated with longer DFS and OS. The basal-like and null subtypes showed similarly unfavorable prognosis, and the mixed and claudin low subtypes showed intermediate prognosis, although this was not statistically significant. Previous studies reported the survival of patients with claudin low TNBC as being similar to those with the luminal B, HER-2 enriched, and basal-like subtypes (Prat et al., 2010).

Another new finding in this study is the pathologic findings of lymphocytic infiltration and tumor cell discohesiveness being related to patient prognosis. Although there is limited information regarding the potential contribution of the presence of lymphocytic infiltration and tumor cell discohesiveness in breast cancer, especially with regard to patient prognosis, it has been suggested that presence of lymphocytic infiltration in tumor tissues may predict complete pathologic response to neoadjuvanat therapy (Denkert et al., 2010; West et al., 2011). Since lymphocytic infiltration as an anticancer immune surveillance is usually known to have positive prognostic and predictive impact (Fridman et al., 2011), the tumor infiltration we observed in triple negative cancer in this study may be understood in the same context. However, further study on the potential prognostic implication of this phenomenon should be sought, considering that we did not perform a detailed analysis on constitutes of inflammatory cells and distribution and cytokine profiling of lymphocytic infiltration. In this study, tumor cell discohesiveness was significantly associated with the overall survival of patients with TNBC in the multivariate analysis. The finding seems closely related to the previously described "tumor budding" in other cancers, especially in colorectal carcinoma. It was defined as single cells or small clusters observed within the stromal tissue at the invasive margin and was reported to be related to prognosis and clinical behavior in colon, lung and oral cavity (Yamaguchi et al., 2010; Wang et al., 2011) It is understood that "tumor budding" is the histopathological reflection of the dynamic process of epithelial-mesenchymal transition (EMT). Considering that one of the most characteristic findings in EMT is known as the loss of cell to cell cohesiveness due to the loss of cell junction of epithelial cells, further evaluation on the EMT related pathway in triple negative cancer should be followed to elucidate the biological meaning of this specific pathologic finding.

We tried TNBC subtyping based on the immunophenotypes of TNBC. Compared to gene expression profiling, this approach may have some limitations. One of the crucial limitations is the absence of the standard criteria of surrogate IHC markers for classification. Thus, it is difficult to assess whether suggested surrogate IHC markers reflect defined molecular subtypes of TNBC. The lack of a standard definition of positive criteria for interpretation of an individual marker makes it even more difficult to assess their subtypes. Because the consensus on the choice of IHC panel has not been made even in the well-studied basal-like type, further studies on the choice of surrogate IHC markers for the classification, as well the standardized criteria for interpretation of positivity, should be performed. Nevertheless, immunohistochemical analysis offers a "practical" alternative to gene profiling for clinical use. Another potential caveat in this study was the limited number of observations in certain groups, since this limited number may lead to false negatives. In addition, a multitude of the statistical tests may result in false positive.

Even with these limitations, the significance of this study is in attempting the molecular subclassification of TNBC using proposed surrogate IHC markers. We confirmed the heterogeneous characteristics of TNBC, since TNBCs may be classified into subtypes based on their expression of surrogate IHC markers, which leads to another question, as to whether there is any specific clinical implication, such as treatment response to a certain therapy, based on the subtypes in this study.

In conclusion, we classified TNBC into basal-like,

claudin low, and molecular apocrine subtypes by immunohistochemical staining for CK5/6, EGFR, claudin 3, claudin 4, claudin 7, E-cadherin, AR, and GGT1. In addition, we described a mixed TNBC subtype showing characteristics of two of the defined types and a null subtype that did not correspond to any of the subtypes.

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