Attempt towards a novel classification of triple-negative breast cancer using immunohistochemical markers

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Abstract. Significant efforts have been made to gain a better understanding of the heterogeneity of triple-negative breast cancers from the histological to the molecular and genomic levels. In this study, we attempted to bring forward gene expression subtypes of triple-negative breast cancer (TBNC) to the clinic, by translating gene stratification to clinically accessible immunohistochemical (IHC) classification. Using IHC analysis, we categorized 154 TBNC cases into three main subclasses. Differences in the frequencies of basic characteristics and clinicopathological parameters between the subtypes were examined using Chi-square tests. We defined three main groups among the 154 triple-negative cases. The basal-like (BL) group expressed cytokeratin (CK) 5/6 and/or CK14 (83 cases), the AR^+ group demonstrated positivity for androgen receptor (18 cases), and the final group exhibited a $CD44^+CD24^{-/low}$ phenotype (39 cases). There were three overlapping cases between the BL subgroup and the $CD44^+CD24^{-/low}$ phenotype subgroup, and 11 unclassified cases. In this new IHC classification, three subcategories exhibited a statistical difference with regard to age, tumor size, histological grade, tumor necrosis, Ki67 labeling index, relapse-free survival, breast cancer-specific survival and response to chemotherapy. According to our definition, the BL group and $CD44^+CD24^{-/low}$ phenotype could be observed in tumors that were not triple-negative, and BL tumors that were triple-negative demonstrated almost undistinguishable clinicopathological characteristics compared with BL tumors that were not triple-negative. The same observation was made with $CD44^+CD24^{-/low}$ tumors that were triple-negative vs. $CD44^+CD24^{-/low}$ tumors that were not. The AR^+ group demonstrated undistinguishable clinicopathological characteristics compared with the luminal subtype. We successfully distinguished three subtypes exhibiting diverse clinicopathological and prognostic characteristics with the minimum use of IHC markers.

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Introduction

Triple-negative breast cancer (TNBC), an aggressive type of breast cancer, lacks effective targeted therapy due to the absence of hormone receptors and human epidermal growth factor-2 (HER2). Therefore, considerable effort has been made to identify subclasses of TNBC with distinct characteristics that may potentially be targeted in the clinic.

In 2008, Cheang *et al* (1) revealed that TNBC cases that positively expressed epidermal growth factor receptor (EGFR) or cytokeratin (CK) 5/6 demonstrated a shorter survival time and poorer response to chemotherapy, but might benefit from EGFR-targeted therapy (2-7). Another marker in TNBC with potential prognostic and therapeutic value, androgen receptor (AR), has drawn particular attention since 2010 (8). In recent years, studies have progressed to the molecular level. Prat *et al* (9) investigated the correlation between TNBC molecular subtypes and the PAM50 intrinsic subtypes as well as the claudin-low subtype. These authors observed that the majority of TNBCs were either basal-like (39 to 54%) or claudin-low (25% to 39%), followed by HER2-enriched and luminal. However, Lehmann *et al* (10) reported another classification based on gene expression profiles of 587 TNBCs: basal-like 1 (BL1), basal-like 2 (BL2), immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL), and luminal androgen receptor (LAR). Further analysis narrowed these down to three main groups (BL, mesenchymal-like and LAR), which demonstrated different responses to cytotoxic and targeted therapies.

These apparently different classifications may be related (11). Basal-like in the PAM50 assay encompassed the TNBC BL subtypes defined by Lehmann as well as certain tumors classified as IM and M (10,12). In addition, MSL describes a similar group of claudin-low cancers while LAR shares a number of gene expression features of estrogen receptor (ER)-positive and HER2-enriched cancers (10,12). Thus, despite the lack of consensus, it appears reasonable to predict that there are three basic subtypes within TNBC (11,13-15).

Gene expression-based classification significantly changes our understanding of the heterogeneity of TNBC. However,

it raises the question of how this sophisticated approach can be translated into a practical and clinically accessible diagnostic test, given that gene identification is currently not feasible for large-scale application on routine formalin-fixed paraffin-embedded clinical samples (16). In this study, we adopt the immunohistochemistry (IHC) methodology. We examined the IHC profile of 154 TNBC cases and identified three subtypes exhibiting diverse clinicopathological and prognostic characteristics with the minimum use of biomarkers.

Patients and methods

Patient selection. We collected breast cancer cases with sufficient medical records from the Department of Breast Surgery, China-Japan Union Hospital of Jilin University, China, between January 2006 and November 2014. Inclusion criteria for this study were: i) female; ii) primary stage I-III invasive breast cancer; iii) no neoadjuvant chemotherapy or radiotherapy prior to surgery; iv) breast tissue samples available for study. All of the subjects underwent surgical treatment according to standard treatment protocols. Clinicopathological parameters including age, histological subtype, tumor size, histological grade, nodal status, and presence of lymphovascular invasion and tumor necrosis were noted. The histological subtype and histological grade were assessed in accordance with standard guidelines and confirmed independently by two pathologists from the Department of Pathology at the China-Japan Union Hospital of Jilin University. The median follow-up time was 68 months (range, 2 to 108 months). The study was approved by the ethics committee of Jilin University.

Immunohistochemistry and scoring. Immunohistochemical staining was performed according to the following protocol. Sections from paraffin-embedded tissue microarrays were cut to 4 μm, deparaffinized in xylene and rehydrated through graded alcohols. Microwave epitope retrieval was performed in target retrieval pH 6.0 (Dako, Carpinteria, CA, USA) for ER and HER2, high pH target retrieval for CK5/6 (Dako), or 10 mM citrate buffer (pH 6.0) for 10 min followed by cooling for 15 min at room temperature for claudins.

The following antibodies were used: clone SP1 against ER (1:300 dilution; Dako), clone SP2 against progesterone receptor (PR; 1:250 dilution; Neomarkers, Fremont, CA, USA), clone SP3 against HER2 (1:200 dilution; Neomarkers), clone SP6 against Ki67 (1:200 dilution; Neomarkers), clone D5/16B4 against CK5/6 (1:100 dilution; M7237; Dako), clone LL002 against CK14 (1:20; NCL-LL002; Novocastra, Newcastle upon Tyne, UK), clone E30 against EGFR (1:50; M7239; Dako), clone NCH-38 against E-cadherin (1:50, Dako), clone V9 against vimentin (1:150, Dako), clone Z23.JM against claudin 3 (1:300; Invitrogen Life Technologies, Carlsbad, CA, USA), clone Ab15104 against claudin 4 (1:300, Abcam), clone Ab27287 against claudin 7 (1:400; Abcam, Cambridge, MA), clone AR27 against AR (1:100, NCL-AR-318), clone 156-3C11 against CD44 (1:100, Cell Signaling Technology, Inc., Danvers, MA, USA), and clone Ab2-SN3b against CD24 (1:100 Neomarkers).

Staining results were assessed by two pathologists in a blinded fashion. For ER, PR and AR status, stains were considered positive if at least 1% of tumor nuclei demonstrated positivity, regardless of the intensity (1 to 3+). For HER2 status, stains were considered positive if at least 30% of tumor cells exhibited a cell membrane staining score of 3+. There are no commonly accepted cut-off points reported for EGFR. Membranous EGFR staining in >1% of tumor cells was used as the definition of protein positivity according to the Dako criteria provided in the pharmDx kit instructions. For Ki67, the mean percentage of nuclear positivity was evaluated in a stepwise manner; i.e. 1, 2, 3, 5, 10, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80 and 90%. For CK5/6 and CK14, staining was scored as positive when more than 10% of the tumor cells demonstrated cytoplasmic and/or membranous staining. E-cadherin expression was analyzed semi-quantitatively according to the percentage of cells demonstrating membrane positivity: 0, 0-10%; 1+, 10-30%; 2+, 30-70%; 3+, > 70%. E-cadherin expression was considered positive when scores were ≥2 and negative when scores were ≤1. Any distinct positive staining of the tumor cytoplasm in cancer cells with the vimentin antibody was regarded as positive vimentin expression. Claudin immunoreactivity was assessed based on a combined score of the extension and intensity of membrane expression. The extension was registered as the percentage of positive cells for claudins: 0, 0%; 1+, <25%; 2+, 25-50%; 3+, >50%. The intensity of membrane immunostaining was graded as: 0 (negative); 1 (weak); 2 (moderate); 3 (strong). The two scores were multiplied to give an overall score of 0-9, of which 0 was considered negative, 1-2 was considered weak, 3-6 moderate, and 9 strong staining. Negative and weak expression was considered as low, while moderate and strong were considered high. Tumors with low expression of all three claudins were defined as claudin-low. For CD44 and CD24, stains were scored positive when more than 10% of the tumor cells exhibited membranous staining. We considered a tumor to have a cancer stem cell (CSC) phenotype when the frequency of $CD44^{+}CD24^{-/low}$ cells was more than 10%, as previously described in other studies (17,18). Any discordant scores were reviewed together by the two scorers to obtain a consensus.

Definition of breast cancer subtypes by IHC. Identifying subgroups of TNBC is of significance for a better understanding of this complex disease. By drawing on the work of Prat *et al* (9,13) and Lehmann *et al* (10,12) on gene expression subtypes, we attempted for the first time to classify TNBC into three subsets using IHC markers. Our main aim was to seek IHC surrogates that potentially identify the main three gene expression subtypes. Here are certain noteworthy points from the studies of Prat *et al* and Lehmann *et al*: i) TNBC subtypes defined by Lehmann differentially correlate with the PAM50 intrinsic subtypes (12,13). BL1, BL2, IM and M cases are primarily composed of the BL intrinsic subtype (99%, 95%, 84% and 97%, respectively), while ~50% of MSL cases and none of LAR cases have the BL intrinsic subtype (12). Therefore, the vast majority of non-basal TNBCs are MSL and LAR tumors. In addition, BL1, BL2, IM and M subtypes express higher levels of basal cytokeratin expression (i.e., CK5/6 and CK14), while tumors in the MSL category exhibit significantly lower basal cytokeratin expression and LAR tumors lack basal cytokeratin expression (10,12). ii) LAR shares a number of gene expression features of ER^{+} and HER2-enriched cancers (10). AR protein is highly expressed within the LAR subgroup,

on average >10-fold higher than all other subtypes (10). iii) MSL is characterized by enrichment for gene expression patterns associated with epithelial-to-mesenchymal (ETM) transition (10,12,19). A portion of the MSL subtypes also are enriched for the CSC-like phenotype (10,12,19), and exhibit low expression of tight junction proteins including claudin 3, 4 and 7 (10,12,19), consistent with a group of cancers previously described as claudin-low (9). iv) The three main subtypes (BL, mesenchymal-like and LAR) defined by Lehmann *et al* (10) are concordant with the three main groups previously identified by Prat *et al* (BL, claudin-low and luminal/HER2-enriched) (13) and by Neve *et al* (20) and Kao *et al* (21), based upon cell lines alone (basal A, associated with the ETS pathway and BRCA1 signatures and resembling BL tumors; basal B, exhibiting mesenchymal and stem/progenitor cell characteristics; and luminal, exhibiting an ER signature and resembling luminal A/B tumors).

Together, it appears feasible to translate the gene expression subtypes into three IHC subtypes. Based on the first point above, triple-negative cases which also positively express either CK5/6 or CK14 are referred to as the 'BL' group in this article. Therefore, the BL subgroup in this article likely encompasses the BL1, BL2, IM and M subtypes and a small proportion of the MSL tumors that express basal cytokeratin. Based on the second point, triple-negative cases which also positively express AR are referred to as the AR^+ group. However, selecting IHC marker panels to define the third group is relatively challenging. According to the classification defined by Lehmann *et al*, the majority of the third group consists of MSL tumors that lack basal cytokeratin expression (12), whereas according to Prat *et al* (13), the third group should be claudin-low. Although MSL and claudin-low share certain similar features, they are not synonymous. All MSL tumors are associated with EMT transition (10,11), which is characterized by downregulation of E-cadherin and occludin and induction of mesenchymal marker proteins including vimentin and fibronectin (22-25), while only a portion of MSL cases are claudin-low, enriched in CSC-like features with an absence of claudin proteins. Therefore, in order to distinguish the most appropriate IHC surrogates for the third group, we explored the ETM phenotype (evaluating vimentin and E-cadherin expression), CSC-like phenotype (analyzing CD44 and CD24 expression), and claudin 3, 4 and 7 expression in all triple-negative cases, and then defined the third group as $vimentin^+$ and E-$cadherin^-$; $CD44^+CD24^{-/low}$ phenotype; low expression of all three claudins, respectively. Vimentin and E-cadherin are well-established and widely accepted as markers for EMT (20-23), while $CD44^+CD24^{-/low}$ is a known marker for the CSC-like phenotype (9,21,26,27).

Statistical analysis. Differences in the frequencies of basic characteristics and clinicopathological parameters among breast cancer subtypes were examined using Chi-square tests, or Fisher's exact test in the case of less than five expected cases. Relapse-free survival (RFS) was defined as the time from the date of diagnosis to the date of relapse of breast cancer, including locoregional recurrence and/or distant metastasis. Breast cancer-specific survival (BCSS) was defined as the date of a patient's diagnosis of breast cancer until mortality. Survival times were censored if the primary or underlying cause of mortality was not breast cancer, or if the patient was still alive on December 30, 2014 (the date when the outcome data were collected). Survival curves were obtained using the Kaplan-Meier method and differences in survival among the breast cancer subtypes were assessed by the log-rank test. Prognostic analyses used the Cox regression method. Univariate analyses tested classical clinicopathological features: age (>50 vs. ≤50), pathological tumor size (pT2-3 vs. pT1), lymph node status (positive vs. negative), histological grade (2 or 3 vs. 1), necrosis (marked vs. minimal or absent), Ki67 (>30% vs. ≤30%), adjuvant chemotherapy (performed vs. not performed). The findings were analyzed using SPSS statistical software for Windows, version 18 (SPSS, Inc., Chicago, IL, USA. All statistical tests were two-sided, and $P<0.05$ was considered to indicate a statistically significant difference.

Results

Patient characteristics. There were a total of 2407 breast cancer patients receiving surgery at the China-Japan Union Hospital of Jilin University between January 2006 and November 2014. Among these, 1646 cases that had informative IHC results were included in the study. The median age at diagnosis in the study population was 54 years (range, 23-87 years). Mastectomy was performed in 78.3% of cases (1289/1646), and 21.7% (357/1646) underwent breast conserving surgery. Following surgery, 82.6% (1360/1646) received adjuvant chemotherapy. The remaining 286 (17.4%) patients did not receive any adjuvant systemic chemotherapy. The median follow-up time was 68 months (range, 2 to 108 months). Of the 1646 patients, 154 had triple-negative breast cancer (TNBC). The clinicopathological characteristics and IHC profiles of the TNBC cases and other types of breast cancer (non-TNBC) are presented in Table I. The Chi-square test revealed a statistically significant difference in tumor size, histological grade, tumor necrosis and Ki67 labeling index between TNBC and non-TNBC patients. The two groups also differed in the levels of AR, CK5/6, CK14, EGFR, E-cadherin, vimentin, claudin 3, 4 and 7 expression and $CD44^+CD24^{-/low}$ phenotype (Fig. 1). TNBCs had a statistically larger percentage of tumors that were positive for CK5/6 (57.8%), CK14 (39.6%), EGFR (59.0%), vimentin (44.2%) and $CD44^+CD24^{-/low}$ phenotype (27.3%) compared with non-TNBCs (2.2%, 2.1%, 6.8%, 7.2% and 2.4%, respectively). AR, E-cadherin, and claudins 3, 4 and 7 staining was greater in non-TNBCs (83.2%, 71.7%, 97.6%, 97.2% and 97.4%, respectively), whereas the positivity for these five markers in TNBCs was 11.7%, 43.5%, 68.2%, 74.0% and 72.7% ($P=0.000$).

New IHC classification of TNBC. As described in the Patients and methods section, we defined triple-negative cases which also positively expressed either CK5/6 or CK14 as the BL group, triple-negative cases which also positively expressed AR as the AR^+ group, and respectively defined the third group as $vimentin^+$ and E-$cadherin^-$; $CD44^+CD24^{-/low}$ phenotype; low expression of claudins 3, 4 and 7. A comparison of these three different classifications is shown in Fig. 2. A lower level of overlap was observed between the BL group and the third group when the third group was defined as $CD44^+CD24^{-/low}$ phenotype, and the proportion of unclassified

Table I. Clinicopathological characteristics of TNBC and non-TNBC patients.

Characteristics	TNBC n=154	Non-TNBC n=1492	P-value
Age			0.649
≤50	85	852	
>50	69	640	
Family history of breast cancer			0.131
No	141	1411	
Yes	13	81	
Histological type			
Invasive ductal carcinoma	93	1082	
Invasive lobular carcinoma	5	143	
Medullary carcinoma	13	82	
Metaplastic carcinoma	12	78	
Apocrine carcinoma	13	78	
Others	6	29	
Pathological tumor size			0.002
pT1	58	792	
pT2-3	96	700	
Histological grade			<0.001
1	23	228	
2	34	943	
3	97	321	
Pathological axillary lymph node status			0.326
Negative	91	820	
Positive	63	672	
Lymphovascular invasion			0.707
Absent	116	1103	
Present	38	389	
Necrosis			<0.001
Minimal or absent	89	1368	
Marked	65	124	
Ki67			<0.001
≤30%	72	1125	
>30%	82	367	
AR			<0.001
Negative	136	251	
Positive	18	1241	
CK5/6			<0.001
Negative	65	1459	
Positive	89	33	
CK14			<0.001
Negative	93	1461	
Positive	61	31	
EGFR			<0.001
Negative	94	1390	
Positive	60	102	
E-cadherin			<0.001
Negative	87	422	
Positive	67	1070	
Vimentin			<0.001
Negative	86	1385	
Positive	68	107	
Claudin 3			<0.001
Negative	49	36	
Positive	105	1456	
Claudin 4			<0.001
Negative	40	42	
Positive	114	1450	
Claudin 7			<0.001
Negative	42	39	
Positive	112	1453	
$CD44^+CD24^{-/low}$			<0.001
No	112	1456	
Yes	42	36	
RFS event			
No	104	1208	
Yes	50	284	
Chemotherapy			
No	31	255	
Yes	123	1237	
Mean survival time (95% CI)	86.3 (79.7-93.1)	98.7 (81.2-114.5)	

TNBC, triple-negative breast cancer; AR, androgen receptor; CK, cytokeratin; EGFR, epidermal growth factor receptor; RFS, relapse free survival; CI, confidence interval.

cases was also relatively smaller in this classification model. Therefore, the three subtypes of TNBC designated in this study are the BL group (83 cases), AR^+ group (18 cases), and $CD44^+CD24^{-/low}$ phenotype (39 cases). Eleven cases that were unclassified and three cases that overlapped between the BL group and $CD44^+CD24^{-/low}$ phenotype were excluded in the following study.

The clinicopathological characteristics of each TNBC subtype are shown in Table II. When a difference among the three groups was detected, multiple comparison was carried out to assess where the difference lies. The Chi-square test revealed that the three subcategories exhibited significantly different characteristics in terms of age, tumor size, histological grade, presence/absence of tumor necrosis and Ki67 labeling index. Multiple comparison further demonstrated that the three subtypes differed significantly from each other in histological grade and tumor necrosis, but not in age, tumor size or Ki67 labeling index. The histological grade of the $CD44^+CD24^{-/low}$ subtype was often grade 3 (53.8%) or grade 2 (28.2%), which was lower than tumors in the BL group (grade 3, 81.9%; grade 2, 14.4%), and higher than those in the AR^+ group (grade 3, 16.7%; grade 2, 33.3%). A total of 38.5%

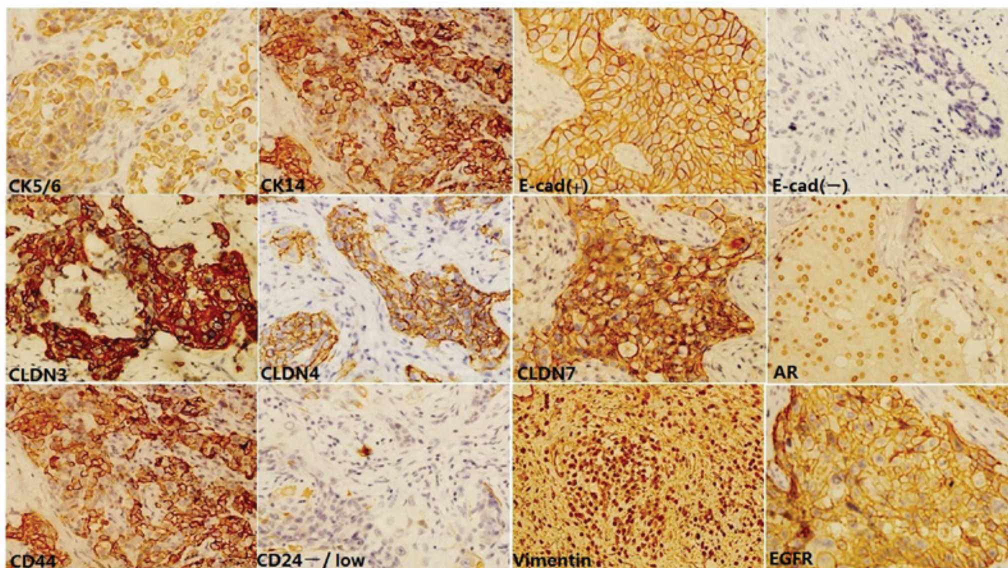

Figure 1. Representative hematoxylin and eosin staining of immunohistochemical biomarkers. Magnification, x200. CK, cytokeratin; E-cad, E-cadherin; CLDN, claudin; AR, androgen receptor; EGFR, epidermal growth factor receptor.

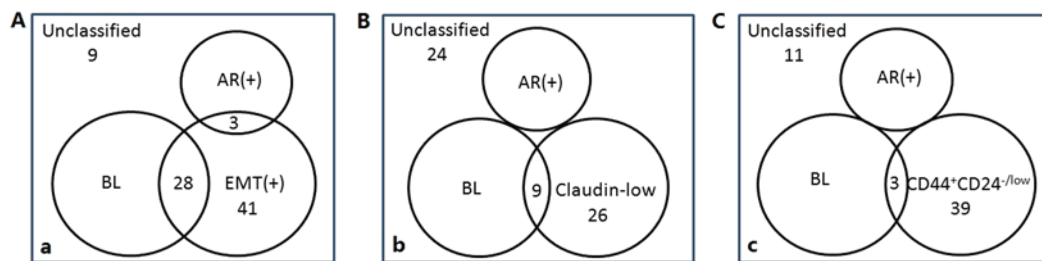

Figure 2. Comparison of three different classifications. (A) The third group is defined as positive for epithelial-to-mesenchymal transition markers vimentin$^+$ and E-cadherin$^-$. (B) The third group is defined as claudin-low: low expression of claudin 3, 4 and 7 (C). The third group is defined as CD44$^+$CD24$^{-/low}$ phenotype. AR, androgen receptor; BL, basal-like; EMT, epithelial-to-mesenchymal transition.

of CD44$^+$CD24$^{-/low}$ subtype cases demonstrated marked tumor necrosis, a percentage intermediate between that of the BL group (57.8%) and the AR$^+$ group (11.1%).

As for age and tumor size, although the Chi-square test revealed a statistically significant difference among the three subcategories, multiple comparison revealed that only the difference between the BL group and AR$^+$ group was significant. Patients with AR$^+$ tumors were older than patients with BL tumors (>50 years, 66.7% vs. 37.3%; multiple comparison test, P=0.0226). A total of 55.5% of AR$^+$ tumors measured ≤2 cm (pT1) while 28.9% of BL tumors were pT1 (multiple comparison test, P=0.0301). In the multiple comparison test, although the CD44$^+$CD24$^{-/low}$ subtype did not reveal distinct characteristics in age and tumor size when separately compared with the BL group and AR$^+$ group, the percentage of patients older than 50 years (53.8%) and the percentage of pT1 tumors (46.2%) were intermediate between the BL group and AR$^+$ group.

As for the Ki67 labeling index, multiple comparison revealed that a significant difference existed between AR+ group and BL group (P<0.001), and also between AR$^+$ group and CD44$^+$CD24$^{-/low}$ group (P=0.0389). However, BL group and CD44$^+$CD24$^{-/low}$ group did not differ in Ki67 (P=0.1463). A total of 22.2% of AR$^+$ tumors had a Ki67 labeling index >30%, which indicated a less proliferative subtype compared with the BL group (65.1%) and CD44$^+$CD24$^{-/low}$ subtype (51.3%).

RFS and BCSS by IHC subtypes. The RFS time of TNBC patients ranged from 4 to 102 months with a median time of 61 months. During the study period, 50 out of 154 (32.5%) TNBC patients experienced local recurrence and/or metastasis. Among these 50 cases, 32 (64%) were in the BL group, 3 (6%) were in the AR$^+$ group, 13 (26%) were in the CD44$^+$CD24$^{-/low}$ subtype, and 2 (4%) were in the unclassified group. The hazard ratio (HR) and 95% confidence interval (CI) of RFS for several basic characteristics by TNBC subtype are shown in Table III. Survival analyses are demonstrated in Fig. 3A. Larger tumor size, positive lymph node status and higher histological grade significantly increased the recurrence risk of TNBC tumors. All of the three subgroups maintained this feature of TNBCs. However, marked tumor necrosis, which could increase the recurrence risk of TNBC, AR$^+$ and CD44$^+$CD24$^{-/low}$ subgroups, did not significantly affect the RFS within the BL subgroup. A higher Ki67 labeling index (>30%) only increased the recurrence risk of AR$^+$ tumors.

The BCSS time ranged from 2 to 108 months with a median time of 68 months. Thirty-six of the 154 (23.4%)

Table II. Clinicopathological characteristics of triple-negative breast cancer immunohistochemical subtypes.

Characteristics	Basal-like n=83	AR^+ n=18	$CD44^+CD24^{-/low}$ n=39	P-value
Age				0.038
≤50	52	6[a]	18	
>0	31	12	21	
Family history of breast cancer				0.839
No	76	17	35	
Yes	7	1	4	
Histological type				
Invasive ductal carcinoma	63	6	14	
Invasive lobular carcinoma	2	1	1	
Medullary carcinoma	11	0	2	
Metaplastic carcinoma	3	0	19	
Apocrine carcinoma	2	11	0	
Others	2	0	3	
Pathological tumor size				0.041
pT1	24	10[a]	18	
pT2-3	59	8	21	
Histological grade				<0.001
1	3	9[a,b]	7[a,b]	
2	12	6	11	
3	68	3	21	
Pathological axillary lymph node status				0.734
Negative	47	12	23	
Positive	36	6	16	
Lymphovascular invasion				0.174
Absent	63	16	26	
Present	20	2	13	
Necrosis				<0.001
Minimal or absent	35	16[a,b]	24[a,b]	
Marked	48	2	15	
Ki67				0.003
≤30%	29	14[a,b]	19[a,b]	
<30%	54	4	20	
EGFR				0.006
Negative	34	8	30[a,b]	
Positive	41	6	9	
E-cadherin				<0.001
Negative	38	4	37[a,b]	
Positive	45	14	2	
Vimentin				<0.001
Negative	55	15	4[a,b]	
Positive	28	3	35	
Claudin 3				<0.001
Negative	10	2	36[a,b]	
Positive	73	16	3	
Claudin 4				<0.001
Negative	9	1	29[a,b]	
Positive	74	17	10	

Table II. Continued.

Characteristics	Basal-like n=83	AR^+ n=18	$CD44^+CD24^{-/low}$ n=39	P-value
Claudin 7				<0.001
Negative	11	1	28[a,b]	
Positive	72	17	11	
Chemotherapy				0.512
No	14	4	10	
Yes	69	14	29	
RFS event				
No	51	15	26	
Yes	32	3	13	
Mean survival time (95% CI)	75.8 (59.9-88.4)	96.3 (84.0-105.7)	84.7 (73.4-94.2)	

[a]Compared with basal-like, P<0.05 [AR^+ vs. basal-like, $CD44^+CD24^{-/low}$ vs. basal-like). [b]Compared with AR^+, P<0.05 [$CD44^+CD24^{-/low}$ vs. AR^+]. AR, androgen receptor; EGFR, epidermal growth factor receptor; RFS, relapse free survival; CI, confidence interval.

TNBC patients succumbed to breast cancer, 12 patients succumbed to other diseases and 106 were alive at the end of the study. The HR and 95% CI for BCSS are shown in Table IV, and survival analyses are shown in Fig. 3B. The three subtypes did not exhibit notable differences either in the RFS or BCSS time (log-rank P=0.053 for RFS, log-rank P=0.126 for BCSS). Multiple comparison only detected a difference between the AR^+ and BL group (log-rank P=0.020 for RFS, log-rank P=0.044 for BCSS). Tumor size, lymph node involvement, histological grade and tumor necrosis were significant prognostic factors in the analysis with all cases of TNBC, and with each subtype of TNBC. In the AR^+ group, a higher Ki67 labeling index (>30%) also demonstrated prognostic value.

Chemotherapy effects on the subtypes. The univariate analyses above tested the prognostic value of adjuvant chemotherapy in all TNBC cases and the different subcategories, and it was revealed that only the BL group received a significant RFS and BCSS benefit from adjuvant chemotherapy (RFS: HR, 0.26; 95% CI, 0.12-0.71; P=0.004; BCSS: HR, 0.18; 95% CI, 0.09-0.65; P<0.001), whereas adjuvant chemotherapy was not associated with significantly prolonged RFS and BCSS in other subtypes and TNBCs as a whole. In order to investigate whether the three subtypes responded differently to chemotherapy, we further divided each subtype into two groups in the survival analysis depending on use of adjuvant chemotherapy. Among the 83 BL patients, 31 were treated with anthracycline-based chemotherapy (19 with doxorubicin/cyclophophamide and 12 with fluorouracil/doxorubicin/cyclophosphamide), 38 were treated with nonanthracycline-based chemotherapy (cyclophosphamide, methotrexate and fluorouracil), and 14 received no adjuvant systemic therapy. Among the 18 AR^+ patients, 9 received anthracycline-based chemotherapy (3 doxorubicin/cyclophosphamide and 6 fluorouracil/doxorubicin/cyclophosphamide), 5 received nonanthracycline-based chemotherapy, and 4 received no adjuvant systemic therapy. Among the 39 $CD44^+CD24^{-/low}$ patients, 21 received anthracycline-based chemotherapy (14 doxorubicin/cyclophosphamide and 7 fluorouracil/doxorubicin/cyclophosphamide), 8 received nonanthracycline-based chemotherapy, and 10 received no adjuvant systemic therapy.

The survival analysis revealed that patients in the BL group without chemotherapy had the shortest RFS and BCSS times and demonstrated a significant survival gain following chemotherapy (P=0.003 for RFS, P<0.001 for BCSS; Fig. 3C-F). Conversely, AR^+ and $CD44^+CD24^{-/low}$ patients did not demonstrate a chemotherapy benefit in either RFS or BCSS. However, the results require careful interpretation due to the small numbers. There was no difference in RFS and BCSS among the three subclasses; however, after we categorized each subclass according to chemotherapy, a notable distinction emerged (log-rank P=0.003 for RFS, log-rank P=0.008 for BCSS).

In the multiple variate analyses (adjusted for age, tumor size, histological grade, lymph node status and tumor necrosis), the BL group demonstrated a significantly poorer survival, with a HR of 2.98 vs. the luminal A cohort (95% CI, 1.38-6.10; P<0.001; Table VA), a higher HR of 3.81 vs. luminal A in the cases without chemotherapy (95% CI, 1.98-6.32; P<0.001; Table VC), and a relatively lower HR of 1.93 vs. luminal A in the cases with chemotherapy (95% CI, 1.21-4.09, P=0.028; Table VB). This confirmed the survival gain of adjuvant chemotherapy in BL patients. In contrast, the AR^+ group did not exhibit a poorer survival vs. the luminal A cohort (HR, 1.38; 95% CI, 0.67-2.14; P=0.204), and the HR was 1.13 (95% CI, 0.62-2.89; P=0.574) in the cases with chemotherapy, and 1.53 (95% CI, 0.68-1.98; P=0.124) in the cases without chemotherapy. The decrease in HR owing to chemotherapy in the AR^+ group (from 1.53 to 1.13) was far less significant than that in the BL group (from 3.81 to 1.93). In the $CD44^+CD24^{-/low}$ group, there was an increase rather than a decrease in HR in the subset of patients who received chemotherapy (HR, 2.30; 95% CI, 0.95-2.84; P=0.003) compared with those who did not (HR, 1.72; 95% CI, 0.88-2.74; P=0.092). We cannot

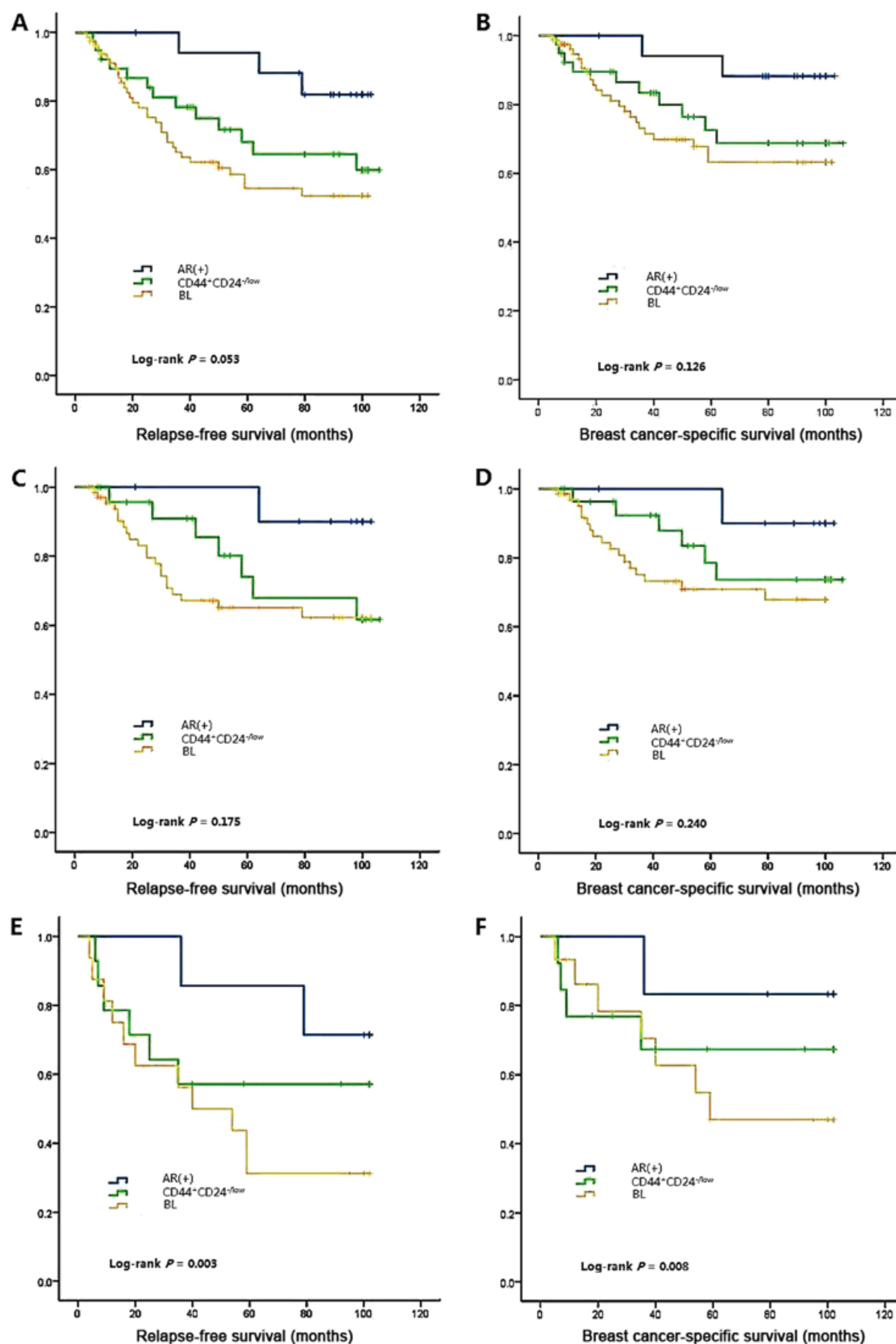

Figure 3. Kaplan-Meier curves of relapse-free and breast cancer-specific survival. (A,C,E) Relapse-free survival according to three immunohistochemistry-based subtypes of triple-negative breast cancer. (B, D and E) Breast cancer-specific survival according to three immunohistochemistry-based subtypes of triple-negative breast cancer. (A and B) all cases combined; (C and D) Cases receiving chemotherapy; (E and F) cases without chemotherapy. AR, androgen receptor; BL, basal-like.

assume that chemotherapy increases the risk of mortality in CD44$^+$CD24$^{-/low}$ phenotype TNBCs, but the results did reveal a notable trait of CD44$^+$CD24$^{-/low}$ tumors in that they do not respond to chemotherapy as well as the BL subtype.

Correlation between IHC TNBC subtypes and subtypes in non-TNBC. CK5/6$^+$, CK14$^+$ and CD44$^+$CD24$^{-/low}$ phenotype were not only observed in TNBCs, but also in non-TNBC cases. Of the 1492 non-TNBCs, 34 cases positively expressed either CK5/6 or CK14, and they are referred to as BL/non-TN in this study. Accordingly, the 36 cases that had the CD44$^+$CD24$^{-/low}$ phenotype are referred to as CD44$^+$CD24$^{-/low}$/non-TN. An issue that cannot be ignored is the correlation between BL tumors that are TNBC (BL/TN) and BL/non-TN, and CD44$^+$CD24$^{-/low}$ tumors that are TNBC (CD44$^+$CD24$^{-/low}$/TN) and CD44$^+$CD24$^{-/low}$/non-TN. To be specific, we take the BL subtype as an example. BL was defined as positive for CK5/6 or CK14, and BL/TN has certain distinct features as shown above, including younger age, higher histological grade and poorer prognosis. Therefore,

Table III. Hazard ratios of triple-negative breast cancer relapse-free survival for several basic characteristics by immunohistochemistry subtypes.

			TNBC subtypes					
	TNBC n=154		Basal-like n=154		AR+ n=154		CD44+CD24-/low n=154	
Variables	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value
Age		1.000		1.000		1.000		1.000
≤50	1.00		1.00		1.00		1.00	
>50	0.88 (0.51-1.52)		0.72 (0.48-1.39)		1.06 (0.78-1.43)		0.92 (0.63-1.5)	
Pathological tumor size		0.012		0.032		0.002		0.019
pT1	1.00		1.00		1.00		1.00	
pT2-3	2.63 (1.54-3.92)		2.26 (1.32-3.49)		3.08 (1.60-4.93)		2.52 (1.22-3.85)	
Lymph node status		0.003		0.024		0.001		0.014
Negative	1.00		1.00		1.00		1.00	
Positive	2.86 (1.62-4.54)		2.41 (1.32-3.54)		3.13 (1.87-4.98)		2.60 (1.43-3.87)	
Histological grade		1.000		1.000		0.048		1.000
1	1.00		1.00		1.00		1.00	
2	1.06 (0.53-2.24)		1.02 (0.43-2.65)		1.26 (1.09-2.87)		1.03 (0.83-1.86)	
3	4,89 (2.37-8.65)	<0.001	3.58 (2.79-6.94)	<0.001	5.19 (2.46-9.12)	<0.001	4.12 (2.03-7.45)	<0.001
Necrosis		0.034		0.193		0.004		0.028
Minimal or absent	1.00		1.00		1.00		1.00	
Marked	2.45 (1.14-3.96)		1.52 (1.06-3.34)		3.06 (1.65-4.12)		2.52 (1.21-3.84)	
Ki67		1.000		1.000		0.046		1.000
≤30%	1.00		1.00		1.00		1.00	
>30%	1.07 (0.56-1.74)		0.88 (0.54-1.45)		1.64 (1.08-2.90)		1.03 (0.84-1.79)	
Adjuvant chemotherapy		1.000		0.004		0.942		0.246
No	1.00		1.00		1.00		1.00	
Yes	0.61 (0.32-1.34)		0.26 (0.12-0.71)		0.89 (0.64-1.35)		2.15 (0.73-6.32)	

TNBC, triple-negative breast cancer; AR, androgen receptor; HR, hazard ratio; CI, confidence interval.

Table IV. Hazard ratios of breast cancer-specific survival in triple-negative breast cancer for several basic characteristics by immunohistochemistry subtypes.

	TNBC subtypes							
	TNBC n=154		Basal-like n=83		AR^+ n=18		$CD44^+CD24^{-/low}$ n=39	
Variables	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value
Age		1.000		1.000		1.000		1.000
≤50	1.00		1.00		1.00		1.00	
>50	1.06 (0.52-1.62)		0.81 (0.38-1.54)		1.10 (0.86-1.52)		0.96 (0.58-1.46)	
Pathological		<0.001		0.016		<0.001		0.010
pT1	1.00		1.00		1.00		1.00	
pT2-3	2.87 (1.64-4.26)		2.44 (1.54-3.98)		3.46 (1.87-5.65)		2.61 (1.31-3.92)	
Lymph node status		<0.001		0.012		<0.001		<0.001
Negative	1.00		1.00		1.00		1.00	
Positive	3.14 (1.81-5.96)		2.53 (1.22-4.66)		3.52 (1.84-5.26)		2.84 (1.38-4.89)	
Histological grade		1.000		0.076				1.000
1	1.00		1.00		1.00		1.00	
2	1.12 (0.63-2.57)		1.36 (0.87-2.84)		1.32 (1.13-3.37)	0.039	1.05 (0.75-1.97)	
3	5.23 (2.74-9.16)	<0.001	3.72 (2.97-7.42)	<0.001	6.21 (2.06-10.26)	<0.001	4.34 (2.42-8.16)	<0.001
Necrosis		0.019		0.042		<0.001		0.008
Minimal or absent	1.00		1.00		1.00		1.00	
Marked	2.53 (1.10-4.13)		1.93 (1.33-4.16)		3.54 (1.97-5.40)		2.83 (1.46-3.99)	
Ki67		1.000		1.000		0.032		1.000
≤30%	1.00		1.00		1.00		1.00	
>30%	1.17 (0.46-1.95)		0.92 (0.67-1.76)		1.76 (1.12-3,23)		1.13 (0.82-1.96)	
Adjuvant chemotherapy		0.084		<0.001		0.803		0.486
Not	1.00		1.00		1.00		1.00	
Yes	0.52 (0.21-0.94)		0.18 (0.09-0.65)		0.92 (0.58-1.42)		1.87 (0.96-4.86)	

TNBC, triple-negative breast cancer; AR, androgen receptor; HR, hazard ratio; CI, confidence interval.

Table V. Cox regression analysis to estimate adjusted hazard ratios of breast cancer subtypes.

A, Cox regression analysis of all 1646 cases

	Relapse-free survival		Breast cancer-specific survival	
Subtypes	HR (95% CI)	P-value	HR (95% CI)	P-value
IHC-Luminal A	1.00		1.00	
IHC-HER2	2.96 (1.23-4.87)	<0.001	3.13 (2.28-5.07)	<0.001
IHC-TNBC	2.04 (1.11-4.38)	0.017	2.15 (1.43-4.16)	0.008
IHC-TN/BL	2.85 (1.18-5.02)	<0.001	2.98 (1.38-6.10)	<0.001
IHC-TN/AR$^+$	1.12 (0.87-1.68)	0.541	1.38 (0.67-2.14)	0.204
IHC-TN/CD44$^+$CD24$^{-/low}$	1.78 (1.07-3.20)	0.073	1.86 (1.18-3.75)	0.052
IHC-TN/unassigned	1.26 (1.11-2.45)	0.296	1.43 (1.06-2.82)	0.187

B, Cox regression analysis of 1360 cases treated with adjuvant chemotherapy

	Relapse-free survival		Breast cancer-specific survival	
Subtypes	HR (95% CI)	P-value	HR (95% CI)	P-value
IHC-Luminal A	1.00		1.00	
IHC-HER2	2.61 (1.24-4.78)	<0.001	2.72 (1.52-5.12)	<0.001
IHC-TNBC	1.93 (1.13-3.34)	0.031	2.11 (1.28-3.84)	0.019
IHC-TN/BL	1.89 (1.17-3.96)	0.048	1.93 (1.21-4.09)	0.028
IHC-TN/AR$^+$	1.09 (0.45-1.56)	0.622	1.13 (0.62-2.89)	0.574
IHC-TN/CD44$^+$CD24$^{-/low}$	2.17 (0.76-2.74)	0.006	2.30 (0.95-2.84)	0.003
IHC-TN/unassigned	1.14 (0.45-2.35)	0.507	1.31 (0.61-2.72)	0.232

C, Cox regression analysis of 286 cases treated without chemotherapy

	Relapse-free survival		Breast cancer-specific survival	
Subtypes	HR (95% CI)	P-value	HR (95% CI)	P-value
IHC-Luminal A	1.00		1.00	
IHC-HER2	3.32 (1.32-5.14)	<0.001	3.48 (1.15-5.54)	<0.001
IHC-TNBC	2.19 (1.11-4.98)	0.002	2.25 (1.08-5.11)	<0.001
IHC-TN/BL	3.64 (1.86-7.65)	<0.001	3.81 (1.98-6.32)	<0.001
IHC-TN/AR$^+$	1.25 (0.76-2.24)	0.287	1.53 (0.68-1.98)	0.124
IHC-TN/CD44$^+$CD24$^{-/low}$	1.68 (1.14-3.07)	0.115	1.72 (0.88-2.74)	0.092
IHC-TN/unassigned	1.37 (0.64-2.73)	0.196	1.48 (0.49-2.64)	0.163

HR, hazard ratio; CI, confidence interval; IHC, immunohistochemistry; HER2, human epidermal growth factor-2; TNBC, triple-negative breast cancer; BL, basal-like; AR, androgen receptor.

through a comparison of clinicopathological characteristics between BL/TN and BL/non-TN we could observe whether these features of CK5/6$^+$ and/or CK14$^+$ tumors would be retained regardless of their clinical ER, PR and HER2 status, and particularly their TN status. The results of comparison may indicate whether BL/TN cases possess these traits more due to their BL status (i.e. their positivity for CK5/6 or CK14) or more due to their TN status (i.e. their triple-negative status). Thus, it may provide us with a better understanding of the intrinsic quality of these TNBC subtypes. A comparison of clinicopathological characteristics between BL/TN and BL/non-TN, and CD44$^+$CD24$^{-/low}$/TN and CD44$^+$CD24$^{-/low}$/non-TN is shown in Table V. As for the AR$^+$ group, we did not compare AR$^+$ tumors that were triple-negative with those that were not. As mentioned in the Patients and methods section, several previous studies (10,13,20,21) have made the same observation that TN tumors with high AR protein and/or gene expression (or LAR, according to Lehmann *et al* (10,12) were usually identified as HER2 or luminal by PAM50 intrinsic subtyping, and their levels of AR

Table VI. Correlation between the triple-negative and non-triple negative breast cancer immunohistochemistry subtypes.

Variables	BL/ TN	BL/ TN	P-value	$CD44^+$ $CD24^{-/low}$/ TN	$CD44^+$ $CD24^{-/low}$/ non-TN	P-value	AR^+/ TN	Luminal	P-value	AR^+/ TN	HER2	P-value
	n=83	n=34		n=39	n=36		n=18	n=965		n=18	n=344	
Age			0.834			0.926			0.818			0.073
≤50	52	22		18	17		6	347		6	189	
>50	31	12		21	19		12	618		12	155	
Family history			0.575			0.775						0.924
No	76	30		35	33		17	896		17	323	
Yes	7	4		4	3		1	69		1	21	
Histological type												
Invasive ductal carcinoma	63	21		14	15		6	872		6	319	
Invasive lobular carcinoma	2	3		1	2		1	49		1	11	
Medullary carcinoma	11	6		2	0		0	12		0	7	
Metaplastic carcinoma	3	1		19	18		0	10		0	0	
Apocrine carcinoma	2	0		0	0		11	14		11	0	
Others	2	3		3	1		0	8		0	7	
Pathological tumor size			0.790			0.378						0.031
pT1	24	9		18	13		10	480		10	107	
pT2-3	59	25		21	23		8	485		8	237	
Histological grade			0.764			0.836			0.561			0.019
1	3	2		7	5		9	363		9	74	
2	12	6		11	12		6	396		6	166	
3	68	26		21	19		3	206		3	104	
Lymph node status			0.609			0.345			0.710			0.318
Negative	47	21		23	25		12	602		12	188	
Positive	36	13		16	11		6	363		6	156	

Table VI. Continued.

	BL/ TN	BL/non-TN		$CD44^+$ $CD24^{-/low}$/ TN	$CD44^+$ $CD24^{-/low}$/ non-TN		AR^+/ TN	Luminal		AR^+/ TN	HER2	
Variables	n=83	n=34	P-value	n=39	n=36	P-value	n=18	n=965	P-value	n=18	n=344	P-value
Lymphovascular invasion			0.787			0.798			0.964			0.808
Absent	63	25		26	21		16	861		16	299	
Present	20	9		13	15		2	104		2	45	
Necrosis			0.553			0.680			0.424			0.145
Minimal or absent	24	8		22	22		15	725		15	230	
Marked	59	26		17	14		3	240		3	114	
Ki67			0.374			0.711			0.489			0.004
≤30%	29	9		19	16		14	678		14	149	
>30%	54	25		20	20		4	287		4	195	
EGFR			0.728			0.701			0.701			<0.001
Negative	41	18		30	29		8	473		8	281	
Positive	42	16		9	7		10	492		10	63	
E-cadherin			0.321			0.192			0.621			0.499
Negative	38	19		37	31		4	265		4	102	
Positive	45	15		2	5		14	700		14	242	
Vimentin			0.650			0.261			0.596			0.747
Negative	55	24		4	7		15	754		15	296	
Positive	28	10		35	29		3	211		3	48	
Claudin 3			0.118			0.611			0.906			0.898
Negative	10	8		36	32		2	116		2	35	
Positive	73	26		3	4		16	849		16	309	
Claudin 4			0.318			0.834			0.647			0.646
Negative	9	6		29	26		1	83		1	12	
Positive	74	28		10	10		17	882		17	332	
Claudin 7			0.827			0.233			0.771			0.458
Negative	11	4		28	30		1	71		1	9	
Positive	72	30		11	6		17	894		17	335	

Table VI. Continued.

Variables	BL/TN	BL/non-TN	P-value	$CD44^+CD24^{-/low}$/TN	$CD44^+CD24^{-/low}$/non-TN	P-value	AR^+/TN	Luminal	P-value	AR^+/TN	HER2	P-value
	n=83	n=34		n=39	n=36		n=18	n=965		n=18	n=344	
RFS event			0.928			0.602			0.714			0.151
No	51	22		26	25		15	857		15	231	
Yes	32	12		13	11		3	108		3	113	
Mean survival time	75.8	77.7		84.7	86.1		96.3	103.2		96.3	79.3	
95% CI	59.9-88.4	62.8-91.4		73.4-94.2	75.6-96.6		84.0-105.7	93.3-109.2		84.0-105.7	60.5-93.6	

BL, basal-like; TN, triple-negative; AR, androgen receptor, HER2, human epidermal growth factor-2; RFS relapse free survival; CI, confidence interval.

expression resembled the levels observed in HER2 and ER-positive tumors that were not TN. However, the authors had divergent opinions on percentage that the HER2 and luminal groups accounted for. According to Mayer *et al* (19), the LAR subtype is classified as HER2 (74.3%) and luminal (14.3%); however, based on the statistics of Lehmann *et al* (10) 82% of LAR cases were luminal (either luminal A or B). Therefore, we separately compared the AR^+ group of TNBC with the luminal subtype [including luminal A (ER^+ and/or PR^+, $HER2^-$) and luminal B (ER^+ and/or PR^+, $HER2^+$)] and HER2 subtype (ER^-, PR^-, $HER2^+$) that were not TNBC. Based on the data from Table VI, BL/TN cases demonstrated almost undistinguishable clinicopathological characteristics compared with BL/non-TN cases, as did $CD44^+CD24^{-/low}$/TN cases compared with $CD44^+CD24^{-/low}$/non-TN cases. The features of the AR^+ group resembled those of the non-TNBC luminal group rather than those of HER2. Next, a survival analysis was performed and differences in RFS and BCSS were compared between BL/TN and BL/non-TN, $CD44^+CD24^{-/low}$/TN and $CD44^+CD24^{-/low}$/non-TN, and the AR^+ and luminal group (Fig. 4). Multiple comparison revealed no significant difference between BL/TN and BL/non-TN (log-rank P=0.9 for RFS, log-rank P=0.9 for BCSS), $CD44^+CD24^{-/low}$/TN and $CD44^+CD24^{-/low}$/non-TN (log-rank P=0.6 for RFS, log-rank P=0.5 for BCSS), or the AR^+ group and luminal group (log-rank P=0.7 for RFS, log-rank P=0.8 for BCSS).

Discussion

In this study, a large number of clinical breast cancer cases were evaluated and the following observations concerning TN breast cancers were made: i) TN disease is a heterogeneous clinical entity composed of three main IHC subtypes, with the BL tumor type predominating (>50%); ii) The three subcategories demonstrated a statistically significant difference with regard to age, tumor size, histological grade, tumor necrosis, Ki67 labeling index and response to chemotherapy; iii) Basal-like tumors that are TN exhibit almost undistinguishable clinicopathological characteristics compared with BL tumors that are non-TN. The same applies with $CD44^+CD24^{-/low}$/TN vs. $CD44^+CD24^{-/low}$/non-TN and AR^+/TN vs. luminal/non-TN.

Our study is a preliminary attempt to use gene expression subtypes in a practical and clinically accessible diagnostic test. We use IHC methodology to observe how TNBC can be broken down into components. This novel IHC classification system was based on the perspectives of Lehmann *et al* (10,12,19), who identified six subtypes (BL1, BL2, IM, M, MSL and LAR), and Prat *et al* (13), who contended that the three main subtypes were BL, claudin-low and luminal/HER2-enriched. These two seemingly different classifications are correlated; for instance, LAR shares a number of gene expression features of luminal and HER2-enriched cancers, as illustrated in Patients and methods. However, in the definition of Prat *et al* (13), the identification of luminal/TN tumors, HER2/TN tumors might appear at first glance to be counterintuitive, and an explanation is required with regard to the discrepancy between gene expression and IHC-based assays. One possibility is the false positivity or false negativity of the IHC-based assays in determining hormone receptor or HER2 status (28). Another possibility is that the pathology and gene expression data

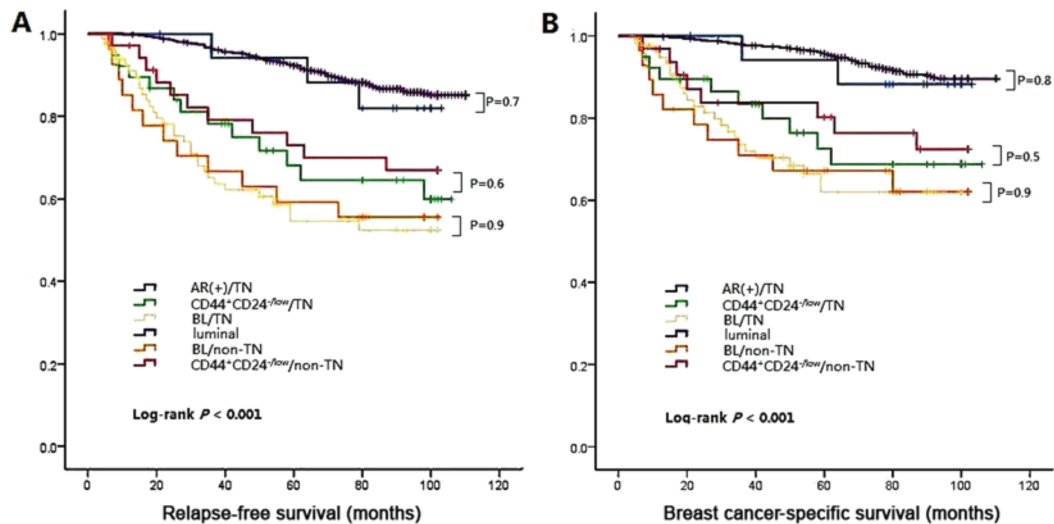

Figure 4. Comparison of relapse-free survival and breast cancer-specific survival between triple-negative (TN) subtypes and non-TN breast cancer subtypes. (A) Relapse-free survival of TN and non-TN subtypes (B). Breast cancer-specific survival in TN and non-TN subtypes. AR, androgen receptor; BL, basal-like.

could have been obtained from two different areas of the same tumor (i.e., intratumor heterogeneity) (29). The most plausible explanation is that gene expression measures a large number of related genes, compared with the three individual pathology-based biomarkers that define TN disease (13). Thus, multigene expression data using tens to hundreds of genes might better capture the true biological profile of a given tumor compared with three or four individual biomarkers (30). For example, a TN tumor that has low levels of ESR1 and PGR, and consequently is ER$^-$ and PR$^-$ by IHC, might be identified as luminal due to the high expression of other luminal-related genes (i.e., *AR, GATA3* and/or *FOXA1*) and the low expression of basal- and proliferation-related genes. Another example comes from the identification of HER2-enriched/TN tumors that do not amplify ERBB2, some of which might be driven by high EGFR (13).

In previous studies, BL breast cancers accounted for up to 15% of all breast cancers (31,32). Most of them used the definition of Nielsen *et al* (31), which is positive staining for CK5/6 or EGFR (31). In this study, the proportion of BL breast cancers was 7.11% (53.9% of TNBCs). Of a total of 117 BL breast cancers, 70.94% (83 of 117) were TNBCs, and 29.06% (34 of 117) were non-TNBCs. We used basal markers CK5/6 and CK14 instead of CK5/6 and EGFR for four reasons: i) Based on the recent progress in TNBC gene subtyping by Prat *et al* (13), expression of EGFR was observed to be significantly increased in HER2-enriched/TN tumors compared with HER2-enriched/non-TN tumors, thus suggesting that certain HER2-enriched tumors, which are at gene level in line with HER2-enriched tumors but are HER2$^-$ by IHC, may be driven by EGFR as discussed above. This implies that EGFR expression is not confined to BL cancers (10,19). ii) The EGFR gene is not enriched in all BL tumors but in the BL2 subtype alone (10). It is also enriched in a minority of mesenchymal subtypes (10). iii) Not only has specificity of EGFR for defining BL breast cancers become lower than it used to be when subtyping was not as comprehensive as today, but also the prognostic value of EGFR was challenged. In the study of Choi *et al* (33), CK5/6 was a poor prognostic marker whereas EGFR was not. 4) According to Won *et al* (34), in a survey of IHC biomarkers for BL breast cancer against a gene expression profile gold standard, CK14 was the most specific (specificity 100%) among the 46 biomarkers surveyed. If we used CK5/6 and EGFR, the proportion of BL breast cancers increased to 14.12%, and accounted for 65.6% of TNBCs, which was similar to the figure of 15% obtained in the previous study. However, this might obscure certain significant information since EGFR$^+$ tumors comprise part of the AR$^+$ group. Indeed, 10 out of 18 AR$^+$ TNBCs demonstrated weak or strong positivity for EGFR.

AR$^+$ tumors constitute a distinct subgroup of TNBC. A total of 11.7% of TNBCs were AR$^+$ in this study. Among 22 studies summarized in the article of Safarpour *et al* (35), the proportion of tumors with positive AR among TNBCs ranged from 6.6% to 75%. Among six studies which had used the most recent ASCO/CAP guidelines (1% and more) for ER, PR and AR positivity, the expression rate of AR ranged from 12.7% to 41.4%. This group has certain valuable clinicopathological features including smaller tumor size, higher median age, lower histological grade, higher percentage of apocrine morphology, lower proliferation index (measured by Ki67) and statistically longer disease-free survival and overall survival (8,36-48). Our study also arrived at similar conclusions. In terms of IHC features, it is noteworthy that none of the AR$^+$ tumors in our study were positive for CK5/6 or CK14, and due to the relatively small series of analyzed samples this may be coincidental; however, it is in accordance with the study of Lehmann *et al* that LAR cancers lacked expression of basal cytokeratins. So far, no organization has recommended AR assessment for breast cancers; however, we support routine assessment of AR at least for TNBCs considering the predictive value of AR in TNBC.

AR$^+$ and BL are two subtypes that have received significant interest and are relatively well analyzed. However, emerging data imply that TN disease is a broad and diverse category for which additional subclassifications are required. One of the contributions of this study is that, for the first time, we distinguished a subgroup of TNBC as the CD44$^+$CD24$^{-/low}$

phenotype using IHC markers, and the overlap between this third group and BL and AR^+ was low (3 and 0 cases, respectively). $CD44^+CD24^{-/low}$ is a marker of breast stem cells and tumor-initiating cells and is observed to be exclusively enriched in claudin-low subtype (26,27). There are also other features in the claudin-low subtype, for instance, low gene expression of tight junction proteins claudin 3, 4 and 7 and E-cadherin (9,26,27,49,50). However, when we used negativity for claudins 3, 4 and 7 to define the third group, there were 24 cases, a relatively large proportion, that could not be classified. This is possibly due to the fact that negativity for all claudins is a much stricter restriction compared with $CD44^+CD24^{-/low}$. In addition, a study of Prat *et al* (9), a researcher who contributed significantly to our knowledge of the claudin-low subtype of breast cancer, revealed that BL tumors did not demonstrate significantly lower expression of CD24 as a group. This crucial distinction may explain the lowest overlap between the BL group and $CD44^+CD24^{-/low}$ group. In another classification where $vimentin^+$ and $E\text{-}cadherin^-$ were used, the highest overlap was observed. In fact, undifferentiated levels of mesenchymal (vimentin) markers exist not only within the claudin-low subtype, but also in BL breast cancers, and no statistically significant difference was observed between claudin-low and BL tumors (9). We defined a number of differences between the $CD44^+CD24^{-/low}$ subtype and the other groups. Clinicopathological characteristics including histological grade and tumor necrosis were different from the AR^+ as well as the BL group. The age at diagnosis of this group was older, the tumor size was smaller, and the Ki67 labeling index was lower than that of the BL group. The two groups demonstrated an unfavorable clinical outcome; however, the $CD44^+CD24^{-/low}$ group did not benefit from adjuvant chemotherapy to the extent that the BL group did. Sabatier *et al* (51) also made similar findings in their study of clinical, pathological and prognostic characterization of claudin-low breast cancers, revealing that the percentage of patients older than 50, the percentage of grade 3 claudin-low tumors, the percentage of tumors measuring 2 cm or less, and the 5-year disease-free survival rate were all intermediate between that of the highly proliferative subtypes (BL and HER2-enriched) and that of less proliferative ones (luminal A and normal).

Without chemotherapy, the BL subcategory had the poorest prognosis in terms of RFS and BCSS. Notably, the BL group demonstrated a distinct clinical benefit with standard adjuvant chemotherapy. Conversely, adjuvant chemotherapy demonstrated little clinical benefit for the AR^+ and $CD44^+CD24^{-/low}$ subclasses. Masuda *et al* (52) performed a retrospective analysis on 130 TNBC cases treated with neoadjuvant adriamycin/cytoxan/taxol-containing chemotherapy, and subtype-specific responses differed substantially, with the BL1 subtype achieving the highest pathological complete remission rate (52%), and the BL2, LAR and MSL subtypes having the lowest responses (0%, 10% and 23%, respectively). In accordance with the work of Masuda *et al* (52), Mayer *et al* (19) observed a similar distribution of subtype-specific differences in survival. These findings should guide differential use of chemotherapy-based regimens and instruct clinical trials to investigate targeted therapies.

In summary, TNBC is a relatively uncommon, notably aggressive disease, and there is a major requirement to better decipher the heterogeneity of TNBC in order to tackle the challenges in combatting this disease. New therapeutic strategies for TNBC are emerging since gene subtyping was identified. Therefore, our future clinical trial design for TNBC intends to focus on continued efforts to translate genetic approaches into clinical utility, to develop a more standard IHC classification of TNBC. Our aim is to provide a labor- and timesaving method for clinicians to distinguish the subtypes of TNBC in their daily work and, in the near future, select a more appropriate personalized therapy based on these subtypes.

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References

1. Cheang MC, Voduc D, Bajdik C, Leung S, McKinney S, Chia SK, Perou CM and Nielsen TO: Basal-like breast cancer defined by five biomarkers has superior prognostic value than triple-negative phenotype. Clin Cancer Res 14: 1368-1376, 2008.
2. Aguiar FN, Mendes HN, Cirqueira CS, Bacchi CE and Carvalho FM: Basal cytokeratin as a potential marker of low risk of invasion in ductal carcinoma in situ. Clinics (Sao Paulo) 68: 638-643, 2013.
3. Fadare O, Wang SA and Hileeto D: The expression of cytokeratin 5/6 in invasive lobular carcinoma of the breast: evidence of a basal-like subset? Hum Pathol 39: 331-336, 2008.
4. Lee HJ, Seo AN, Kim EJ, Jang MH, Kim YJ, Kim JH, Kim SW, Ryu HS, Park IA, Im SA, *et al*: Prognostic and predictive values of EGFR overexpression and EGFR copy number alteration in HER2-positive breast cancer. Br J Cancer 112: 103-111, 2015.
5. Park HS, Jang MH, Kim EJ, Kim HJ, Lee HJ, Kim YJ, Kim JH, Kang E, Kim SW, Kim IA and Park SY: High EGFR gene copy number predicts poor outcome in triple-negative breast cancer. Mod Pathol 27: 1212-1222, 2014.
6. Rakha EA, El-Sayed ME, Green AR, Lee AH, Robertson JF and Ellis IO: Prognostic markers in triple-negative breast cancer. Cancer 109: 25-32, 2007.
7. Sood N and Nigam JS: Correlation of CK5 and EGFR with clinicopathological profile of triple-negative breast cancer. Patholog Res Int 2014: 141864, 2014.
8. Niemeier LA, Dabbs DJ, Beriwal S, Striebel JM and Bhargava R: Androgen receptor in breast cancer: expression in estrogen receptor-positive tumors and in estrogen receptor-negative tumors with apocrine differentiation. Mod Pathol 23: 205-212, 2010.
9. Prat A, Parker JS, Karginova O, Fan C, Livasy C, Herschkowitz JI, He X and Perou CM: Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. Breast Cancer Res 12: R68, 2010.
10. Lehmann BD, Bauer JA, Chen X, Sanders ME, Chakravarthy AB, Shyr Y and Pietenpol JA: Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. J Clin Invest 121: 2750-2767, 2011.
11. Turner NC and Reis-Filho JS: Tackling the diversity of triple-negative breast cancer. Clin Cancer Res 19: 6380-6388, 2013.
12. Lehmann BD and Pietenpol JA: Identification and use of biomarkers in treatment strategies for triple-negative breast cancer subtypes. J Pathol 232: 142-150, 2014.
13. Prat A, Adamo B, Cheang MC, Anders CK, Carey LA and Perou CM: Molecular characterization of basal-like and non-basal-like triple-negative breast cancer. Oncologist 18: 123-133, 2013.
14. Abramson VG, Lehmann BD, Ballinger TJ and Pietenpol JA: Subtyping of triple-negative breast cancer: implications for therapy. Cancer 121: 8-16, 2015.

15. Schmadeka R, Harmon BE and Singh M: Triple-negative breast carcinoma: current and emerging concepts. Am J Clin Pathol 141: 462-477, 2014.
16. Choo JR and Nielsen TO: Biomarkers for basal-like breast cancer. Cancers (Basel) 2: 1040-1065, 2010.
17. Ricardo S, Vieira AF, Gerhard R, Leitão D, Pinto R, Cameselle-Teijeiro JF, Milanezi F, Schmitt F, Paredes J. Breast cancer stem cell markers CD44, CD24 and ALDH1: expression distribution within intrinsic molecular subtype. J Clin Pathol 64: 937-946, 2011.
18. Mylona E, Giannopoulou I, Fasomytakis E, Nomikos A, Magkou C, Bakarakos P, Nakopoulou L. The clinicopathologic and prognostic significance of $CD44^{+}/CD24^{(-/low)}$ and $CD44^{-}/CD24^{+}$ tumor cells in invasive breast carcinomas. Hum Pathol 39:1096-1102, 2008
19. Mayer IA, Abramson VG, Lehmann BD and Pietenpol JA: New strategies for triple-negative breast cancer - deciphering the heterogeneity. Clin Cancer Res 20: 782-790, 2014.
20. Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, Fevr T, Clark L, Bayani N, Coppe JP, Tong F, *et al*: A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer Cell 10: 515-527, 2006.
21. Kao J, Salari K, Bocanegra M, Choi YL, Girard L, Gandhi J, Kwei KA, Hernandez-Boussard T, Wang P, Gazdar AF, *et al*: Molecular profiling of breast cancer cell lines defines relevant tumor models and provides a resource for cancer gene discovery. PLoS One 4: e6146, 2009.
22. Liu T, Zhang X, Shang M, Zhang Y, Xia B, Niu M, Liu Y and Pang D: Dysregulated expression of Slug, vimentin and E-cadherin correlates with poor clinical outcome in patients with basal-like breast cancer. J Surg Oncol 107: 188-194, 2013.
23. Lee J, Hahm ER, Marcus AI and Singh SV: Withaferin A inhibits experimental epithelial-mesenchymal transition in MCF-10A cells and suppresses vimentin protein level in vivo in breast tumors. Mol Carcinog 54: 417-429, 2015.
24. Rito M, Schmitt F, Pinto AE and André S: Fibromatosis-like metaplastic carcinoma of the breast has a claudin-low immunohistochemical phenotype. Virchows Arch 465: 185-191, 2014.
25. Zhang Y, Toy KA and Kleer CG: Metaplastic breast carcinomas are enriched in markers of tumor-initiating cells and epithelial to mesenchymal transition. Mod Pathol 25: 178-184, 2012.
26. Giatromanolaki A, Sivridis E, Fiska A and Koukourakis MI: The CD44+/CD24- phenotype relates to 'triple-negative' state and unfavorable prognosis in breast cancer patients. Med Oncol 28: 745-752, 2011.
27. Honeth G, Bendahl PO, Ringner M, Ringnér M, Saal LH, Gruvberger-Saal SK, Lövgren K, Grabau D, Fernö M, Borg A and Hegardt C: The CD44+/CD24-phenotype is enriched in basal-like breast tumors. Breast Cancer Res 10: R53, 2008.
28. Hammond ME, Hayes DF, Wolff AC, Mangu PB and Temin S: American Society of Clinical Oncology/College of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer. J Oncol Pract 6: 195-197, 2010.
29. Barry WT, Kernagis DN, Dressman HK, Griffis RJ, Hunter JD, Olson JA, Marks JR, Ginsburg GS, Marcom PK, Nevins JR, *et al*: Intratumor heterogeneity and precision of microarray-based predictors of breast cancer biology and clinical outcome. J Clin Oncol 28: 2198-2206, 2010.
30. Prat A, Parker JS, Fan C and Perou CM: PAM50 assay and the three-gene model for identifying the major and clinically relevant molecular subtypes of breast cancer. Breast Cancer Res Treat 135: 301-306, 2012.
31. Nielsen TO, Hsu FD, Jensen K, Cheang M, Karaca G, Hu Z, Hernandez-Boussard T, Livasy C, Cowan D, Dressler L, *et al*: Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma. Clin Cancer Res 10: 5367-5374, 2004.
32. Badve S, Dabbs DJ, Schnitt SJ, Baehner FL, Decker T, Eusebi V, Fox SB, Ichihara S, Jacquemier J, Lakhani SR, *et al*: Basal-like and triple-negative breast cancers: a critical review with an emphasis on the implications for pathologists and oncologists. Mod Pathol 24: 157-167, 2011.
33. Choi YL, Oh E, Park S, Kim Y, Park YH, Song K, Cho EY, Hong YC, Choi JS, Lee JE, *et al*: Triple-negative, basal-like and quintuple-negative breast cancers: better prediction model for survival. BMC Cancer 10: 507, 2010.
34. Won JR, Gao D, Chow C, Cheng J, Lau SY, Ellis MJ, Perou CM, Bernard PS and Nielsen TO: A survey of immunohistochemical biomarkers for basal-like breast cancer against a gene expression profile gold standard. Mod Pathol 26: 1438-1450, 2013.
35. Safarpour D, Pakneshan S and Tavassoli FA: Androgen receptor (AR) expression in 400 breast carcinomas: is routine AR assessment justified? Am J Cancer Res 4: 353-368, 2014.
36. McNamara KM, Yoda T, Miki Y, Chanplakorn N, Wongwaisayawan S, Incharoen P, Kongdan Y, Wang L, Takagi K, Mayu T, *et al*: Androgenic pathway in triple negative invasive ductal tumors: its correlation with tumor cell proliferation. Cancer Sci 104: 639-646, 2013.
37. Tsutsumi Y: Apocrine carcinoma as triple-negative breast cancer: novel definition of apocrine-type carcinoma as estrogen/progesterone receptor-negative and androgen receptor-positive invasive ductal carcinoma. Jpn J Clin Oncol 42: 375-386, 2012.
38. Thike AA, Yong-Zheng Chong L, Cheok PY, Li HH, Wai-Cheong Yip G, Huat Bay B, Tse GM, Iqbal J and Tan PH: Loss of androgen receptor expression predicts early recurrence in triple-negative and basal-like breast cancer. Mod Pathol 27: 352-360, 2014.
39. Tsang JY, Ni YB, Chan SK, Shao MM, Law BK, Tan PH and Tse GM: Androgen receptor expression shows distinctive significance in ER positive and negative breast cancers. Ann Surg Oncol 21: 2218-2228, 2014.
40. Pistelli M, Caramanti M, Biscotti T, Santinelli A, Pagliacci A, De Lisa M, Ballatore Z, Ridolfi F, Maccaroni E, Bracci R, *et al*: Androgen receptor expression in early triple-negative breast cancer: clinical significance and prognostic associations. Cancers (Basel) 6: 1351-1362, 2014.
41. McNamara KM, Yoda T, Takagi K, Miki Y, Suzuki T and Sasano H: Androgen receptor in triple negative breast cancer. J Steroid Biochem Mol Biol 133: 66-76, 2013.
42. Gasparini P, Fassan M, Cascione L, Guler G, Balci S, Irkkan C, Paisie C, Lovat F, Morrison C, Zhang J, *et al*: Androgen receptor status is a prognostic marker in non-basal triple negative breast cancers and determines novel therapeutic options. PLoS One 9: e88525, 2014.
43. Loibl S, Müller BM, von Minckwitz G, Schwabe M, Roller M, Darb-Esfahani S, Ataseven B, du Bois A, Fissler-Eckhoff A, Gerber B, *et al*: Androgen receptor expression in primary breast cancer and its predictive and prognostic value in patients treated with neoadjuvant chemotherapy. Breast Cancer Res Treat 130: 477-487, 2011.
44. Micello D, Marando A, Sahnane N, Riva C, Capella C and Sessa F: Androgen receptor is frequently expressed in HER2-positive, ER/PR-negative breast cancers. Virchows Arch 457: 467-476, 2010.
45. Ogawa Y, Hai E, Matsumoto K, Ikeda K, Tokunaga S, Nagahara H, Sakurai K, Inoue T and Nishiguchi Y: Androgen receptor expression in breast cancer: relationship with clinicopathological factors and biomarkers. Int J Clin Oncol 13: 431-435, 2008.
46. Witzel I, Graeser M, Karn T, Schmidt M, Wirtz R, Schütze D, Rausch A, Jänicke F, Milde-Langosch K and Müller V: Androgen receptor expression is a predictive marker in chemotherapy-treated patients with endocrine receptor-positive primary breast cancers. J Cancer Res Clin Oncol 139: 809-816, 2013.
47. Tang D, Xu S, Zhang Q and Zhao W: The expression and clinical significance of the androgen receptor and E-cadherin in triple-negative breast cancer. Med Oncol 29: 526-533, 2012.
48. Hu R, Dawood S, Holmes MD, Collins LC, Schnitt SJ, Cole K, Marotti JD, Hankinson SE, Colditz GA and Tamimi RM: Androgen receptor expression and breast cancer survival in postmenopausal women. Clin Cancer Res 17: 1867-1874, 2011.
49. Lu S, Singh K, Mangray S, Tavares R, Noble L, Resnick MB and Yakirevich E: Claudin expression in high-grade invasive ductal carcinoma of the breast: correlation with the molecular subtype. Mod Pathol 26: 485-495, 2013.
50. Gerhard R, Ricardo S, Albergaria A, Gomes M, Silva AR, Logullo ÂF, Cameselle-Teijeiro JF, Paredes J and Schmitt F: Immunohistochemical features of claudin-low intrinsic subtype in metaplastic breast carcinomas. Breast 21: 354-360, 2012.
51. Sabatier R, Guille A, Adelaide J, Chaffanet M, Viens P, Birnbaum D, Bertucci F and Finetti P: Claudin-low breast cancers: clinical, pathological, molecular and prognostic characterization. Mol Cancer 13: 228, 2014.
52. Masuda H, Baggerly KA, Wang Y, Zhang Y, Gonzalez-Angulo AM, Meric-Bernstam F, Valero V, Lehmann BD, Pietenpol JA, Hortobagyi GN, *et al*: Differential response to neoadjuvant chemotherapy among 7 triple-negative breast cancer molecular subtypes. Clin Cancer Res 19: 5533-5540, 2013.