A triple-negative breast cancer surrogate subtype classification that correlates with gene expression subtypes

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Abstract

Background

Triple-negative breast cancer (TNBC) is a heterogeneous tumor lacking specific therapeutic targets. Several TNBC classifications have been identified using gene expression profiles, but they are difficult to use in clinical practice. In this study, we have developed a TNBC surrogate subtype classification that represents TNBC subtypes based on the Vanderbilt subtype classification.

Methods

This study included patients who underwent primary curative breast cancer surgery for TNBC between January 2009 and October 2017 at Seoul St. Mary's Hospital. Representative formalin-fixed paraffin embedded blocks were used for gene expression analysis and tissue microarray construction for immunohistochemical (IHC) staining. The Vanderbilt subtypes were re-classified into 4 groups: basal-like (BL), mesenchymal-like (M), immunomodulatory (IM) and luminal androgen receptor (LAR) subtype. After IHC staining, classification and regression tree (CART) modeling was applied to develop a surrogate subtype classification.

Results

A total of 145 patients were included in this study. The study cohort was allocated to the Vanderbilt 4 subtypes as LAR (n = 22, 15.2%), IM (n = 32, 22.1%), M (n = 38, 26.2%), BL (n = 25, 17.2%) and unclassified (n = 28, 19.3%). After excluding nine (6.2%) patients due to poor IHC staining quality, CART modeling was performed. TNBC surrogate subtypes were defined as follows: LAR subtype, androgen receptor Allred score 8; IM subtype, LAR-negative with a tumor-infiltrating lymphocyte (TIL) score > 70%; M subtype, LAR-negative with a TIL score < 20%; BL subtype, LAR-negative with a TIL score 20–70% and diffuse, strong p16 staining. The study cohort was classified by the surrogate subtypes as LAR (n = 26, 17.9%), IM (n = 21, 14.5%), M (n = 44, 30.3%), BL1 (n = 27, 18.6%) and unclassified (n = 18, 12.4%). The performance of the surrogate subtypes to predict TNBC Vanderbilt 4 subtypes was good with an accuracy of 0.708. Each subtype exhibited distinct clinicopathologic features, and the M subtype showed a significantly worse survival rate than the other subtypes.

Conclusions

In this study, we have developed a TNBC surrogate subtype classification that correlates with the Vanderbilt subtype adopting AR, TIL and p16. The surrogate subtype classification is a practical and accessible diagnostic test, that can be easily applied in clinical practice.

Background

Triple-negative breast cancer (TNBC) is defined by the lack of estrogen receptor and progesterone receptor, along with the absence of HER2 overexpression or amplification. TNBC constitutes 15–20% of all breast cancers and is characterized by an aggressive clinical course, earlier age of onset, and worse clinical outcomes [1, 2]. Estrogen receptor, progesterone receptor, and HER2 are molecular targets of therapeutic agents that provide improved treatment outcomes in patients with breast cancer. However, patients with TNBC lack targeted therapies and chemotherapy remains the sole established treatment option.

By definition, TNBC is considered a diagnosis of exclusion, which implies that it is a breast cancer subtype comprising heterogeneous tumors [3]. This molecular heterogeneity has resulted in a lack of targeted therapies for TNBC and subsequent failure to achieve improved survival. Thus, a concerted effort is underway to classify this aggressive disease subtype and provide targeted treatment. Several TNBC classifications have been identified based on the results of gene expression profile studies involving unsupervised clustering analyses [4–7]. The TNBC classifications presented in these studies have some common features and subtypes, but do not overlap completely [4–7].

The most commonly used classification method is the Vanderbilt subtype, described by Lehmann and colleagues [4]. Twenty-one publicly available data sets were used to analyze gene expression profiles for 587 TNBC tumors. Six stable molecular subtypes were identified, namely basal-like 1 (BL1), basal-like 2 (BL2), immunomodulatory (IM), luminal androgen receptor (LAR), mesenchymal (M), and mesenchymal stem-like (MSL). In a retrospective study, Masuda et al. demonstrated that the Vanderbilt subtype is an independent predictor of pathologic complete response, implying the potential usefulness of TNBC molecular subtyping in the clinic [8].

TNBC classification based on gene expression profiling is essential for the guidance of individualized treatment. However, gene expression profiling has practical limitations: high cost, a complicated technological process, slow turnaround time, and potential batch effects. These features restrict the application of gene expression-based TNBC subtypes in large clinical trials and routine clinical practice. The identification of a surrogate subtype classification may be an alternative for clinical use because it is much cheaper, faster, and easier to access. In this study, we developed a TNBC surrogate subtype classification that represents TNBC gene expression-based subtypes by using Vanderbilt subtypes.

Methods

Patient selection and ethical approval

Patients who underwent breast cancer surgery between January 2009 and October 2017 at Seoul St. Mary's Hospital were included in this retrospective study. Women who were diagnosed with TNBC and underwent primary curative surgery were initially considered eligible for this study. Only patients who provided written informed consent to participate in gene studies were included in this study. Furthermore, patients with a tumor size < 1 cm and those whose tumors had generated poor-quality formalin-fixed paraffin embedded (FFPE) tissues were excluded to ensure sufficient FFPE tissue for analysis. This study was approved by the institutional review board of Seoul St. Mary's Hospital, College of Medicine, The

Catholic University of Korea (IRB no. KC17TNSI0414), and the requirement for informed consent was waived.

Tissue microarray construction and RNA microarray expression analysis

All FFPE sections were reviewed and representative FFPE blocks were marked for RNA extraction and tissue microarray construction. Four 10- μ m-thick sections were prepared from each representative FFPE block for RNA extraction, and two 2-mm tissue cores per tumor were obtained to construct tissue microarrays. Gene expression analysis was performed using the Affymetrix® Human Gene 2.0 ST Array. The raw data were summarized and normalized with the robust multi-average method implemented in the Affymetrix® Power Tools.

Gene expression-based TNBC subtyping

The web-based subtyping tool TNBCtype was used to classify the TNBC cohort into Vanderbilt subtypes [9]. The original Vanderbilt subtype method classifies TNBC into six groups, with three main subtypes: basal-like (BL), mesenchymal-like, and LAR [4]. Lehmann et al. refined their TNBC subtypes from six to four subtypes by re-assigning IM and MSL subtypes because these two subtype calls are strongly influenced by stromal cell gene expression [10]. However, considering the importance of immunotherapy in TNBC, maintenance of the IM subtype is needed for precision medicine treatment of TNBC subtypes. Furthermore, TNBC subtypes from other groups include a subtype categorized according to immune response, highlighting the need to maintain the IM subtype [5–7, 11]. Therefore, we re-classified the Vanderbilt TNBC subtypes into four groups: basal-like (BL), mesenchymal-like (M), immunomodulatory (IM), and luminal androgen receptor (LAR).

Pathology review and immunohistochemical (IHC) staining

Biomarkers and IHC antibodies for each subtype were selected after a thorough literature review with a preference for antibodies that were already used in pathology clinics to reduce IHC staining bias and provide optimal applicability in clinical practice. p16, EGFR, CK5/6, and p53 were selected for identification of the BL subtype [12–17]. The androgen receptor (AR) was identified as a notable biomarker for the LAR subtype in several previous studies [4, 6, 13, 16]. MUC1 overexpression was also reported in LAR subtypes [6]. The IM subtype reportedly shows high expression levels of immune signatures and checkpoint inhibitor genes including PD-L1, tumor-infiltrating lymphocytes (TIL), and CD8 [17]. The M subtype is related to epithelial-mesenchymal transition and is associated with both MUC1 and SMAD4 expression [18, 19].

IHC staining was performed using the tissue microarrays. The details of IHC antibodies are shown in Supplementary Table 1. IHC staining results were assessed in a blinded manner by a pathologist specializing in breast cancer evaluation. AR IHC was evaluated by using the Allred score system [20]. TP53 IHC scoring was based on the proportion of tumor cells with strongly positive nuclear staining, using a grading system of 0%, 1–50%, or > 50%. CK5/6 IHC expression was graded with the proportion of tumor cells that showed any cytoplasmic staining (< 1%, 1–50%, or > 50%). EGFR IHC was graded in accordance with the following system: 0, no membrane staining; 1+, faint, partial membrane staining; 2+, weak,

complete membrane staining in > 10% of invasive cancer cells; and 3+, intense complete membrane staining in > 10% of invasive cancer cells [21]. p16 IHC expression was graded as negative, weak and mosaic pattern, or diffuse and strong. CD8 scoring was performed from the proportion of CD8-positive lymphocytes in the stromal area with score ranges of 0–10%, 11–20%, 20–30%, and > 30%. PD-L1 IHC findings were considered positive when PD-L1-positive immune cells were identified in more than 1% of the tumor area. MUC1 IHC expression was graded on the basis of the proportion of cytoplasmic stained cells with percentage ranges of 0%, 1–10%, 10–50%, and > 50% [22]. SMAD4 IHC findings were evaluated according to the staining intensity of any tumor cells using the following categories: negative, weak, or strong [23]. TILs were measured in accordance with recommendations from the International TILs Working Group 2014 [24]. All tumors were evaluated in two cores and summarized by the average values for TILs and CD8 grading or maximum values for other IHC antibodies.

Statistical analysis

Clinicopathologic features according to TNBC subtypes were compared using the Pearson chi-squared test or analysis of variance. Disease-free survival 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. Overall survival was defined as the time from the date of diagnosis to patient death or last date of outpatient follow-up. Survival curves were generated using the Kaplan–Meier method and differences in survival according to TNBC subtype were calculated using the log-rank test. Multivariate survival analysis was performed using the Cox proportional hazards regression analysis.

TNBC surrogate subtyping according to pathology review and IHC staining results was performed using the classification and regression trees (CART) model [25]. The predictors included IHC results and TIL value. The target variable was the TNBC Vanderbilt 4 subtype, excluding unclassified patients (these patients were entirely excluded from the modeling process). The parameters of CART were set to max depth = 5 and complexity parameter = 0.001. The other parameters were set to default values. The modeling process was performed using the rpart package in R. A novel TNBC surrogate subtype classification was developed based on the CART classification model. The performance of the novel TNBC surrogate subtype classification was estimated by the following parameters: accuracy, specificity, precision, and recall rate. All statistical analysis was performed using R software (R Foundation for Statistical Computing, Vienna, Austria).

Results

Clinicopathologic characteristics

In total, 3,158 patients underwent surgical treatment of breast cancer between January 2009 and October 2017. Among them, 485 patients (15.4%) had TNBC tumors. After exclusion of patients who underwent neoadjuvant chemotherapy, as well as those with very small tumors, insufficient FFPE tissue quality, or lack of informed consent, 147 patients were initially included in this study. Prior to the analysis, two

additional patients were excluded: one was identified as HER2-positive on additional in situ hybridization tests and one was partially estrogen-receptor-positive.

The clinicopathologic characteristics of the patients with TNBC included in this study are shown in Table 1. The median age at diagnosis was 55 years (range, 18–86 years). The majority of the tumors were invasive ductal carcinoma (n = 111, 76.6%), followed by metaplastic carcinoma (n = 16, 11.0%). The stages of cancer were as follows: stage I, 47 patients (32.4%); stage II, 87 patients (60.0%); and stage III, 11 patients (7.6%).

Table 1
Clinicopathologic characteristics of all patients with TNBC

		n (%)
Age	≤ 35	20 (13.8)
	> 35, ≤ 50	36 (24.8)
	> 50	89 (61.4)
Breast operation	BCS	113 (77.9)
	Mastectomy	32 (22.1)
Axilla operation	SLNB	92 (63.4)
	ALND	52 (35.9)
	Not done	1 (0.7)
Histologic subtype	IDCa	111 (76.6)
	ILCa	4 (2.8)
	Metaplastic	16 (11.0)
	Medullary	9 (6.2)
	Apocrine	2 (1.4)
	Adenoid cystic	1 (0.7)
	Micropapillary	1 (0.7)
	Papillary	1 (0.7)
Histologic grade	Grade 2	23 (15.9)
	Grade 3	122 (84.1)
Lymphovascular invasion	No	89 (61.4)
	Yes	56 (38.6)
Tumor size	≤ 2 cm	60 (41.4)
	> 2, ≤ 5 cm	81 (55.9)
	> 5 cm	4 (2.8)

BCS, breast-conserving surgery; SLNB, sentinel lymph node biopsy; ALND, axillary lymph node dissection; IDCa, invasive ductal carcinoma; ILCa, invasive lobular carcinoma

* Cancer staging was performed in accordance with the AJCC 7th staging system.

† Ki67 median value at Seoul St. Mary's Hospital is 25%

		n (%)
N stage	N0	97 (66.9)
	N1	36 (24.8)
	N2–3	12 (8.3)
Stage*	I	47 (32.4)
	II	87 (60.0)
	III	11 (7.6)
Ki67 [†]	< 25%	11 (7.6)
	≥ 25%	134 (92.4)
BCS, breast-conserving surgery; SLNB, sentinel lymph node biopsy; ALND, axillary lymph node dissection; IDCa, invasive ductal carcinoma; ILCa, invasive lobular carcinoma		
* Cancer staging was performed in accordance with the AJCC 7th staging system.		
† Ki67 median value at Seoul St. Mary's Hospital is 25%		

TNBC classification with RNA expression and TIL and IHC staining data

The web-based subtyping tool TNBCtype was used to classify the TNBC cohort into Vanderbilt subtypes on the basis of RNA expression data. The cohort was allocated as follows: BL1, 27 patients (18.6%); BL2, 15 patients (10.3%); IM, 26 patients (17.9%); LAR, 15 patients (10.3%); M, 20 patients (13.8%); MSL, 17 patients (11.7%); and unclassified, 25 patients (17.2%). Vanderbilt subtypes were re-classified into four subtypes (BL, IM, M, and LAR) by re-assigning BL2 and MSL subtypes to the second-highest correlated centroid. After re-classification, the Vanderbilt 4 subtypes were allocated as follows: BL, 25 patients (17.2%); IM, 32 patients (22.1%); M, 38 patients (26.2%); LAR, 22 patients (15.2%); and unclassified, 28 patients (19.3%).

TNBC surrogate subtyping was developed using the CART model to correlate with the Vanderbilt 4 subtypes (Fig. 1, 2) [25]. Among the 145 patients, nine (6.2%) were excluded due to poor IHC staining quality. The fitted CART model is illustrated in Supplementary Fig. 1. The original CART model was modified to apply simple and practically feasible criteria for surrogate subtype classification. The LAR subtype constituted an AR Allred score of 8 (5 + 3). The IM subtype was defined as LAR-negative findings with a TIL score > 70%. The M subtype was defined as LAR-negative findings with a TIL score < 20%. The BL subtype was defined as LAR-negative, IM-negative, and M-negative findings, with a diffuse and strong p16 staining pattern. The remaining manifestations were considered unclassified. The 136 patients were classified as follows: LAR subtype (26 patients, 17.9%), IM subtype (21 patients, 14.5%), M subtype (44 patients, 30.3%), BL1 subtype (27 patients, 18.6%), and unclassified (18 patients, 12.4%). Patients

categorized as unclassified had the M Vanderbilt subtype comprised as the greatest proportion (33.3%). The unclassified patients from the Vanderbilt 4 subtypes and surrogate subtypes were excluded from the assessment of the performance of the established surrogate classification. The performance of the surrogate subtypes to predict TNBC Vanderbilt 4 subtypes was good (Fig. 3). The accuracy was 0.708, specificity was 0.922, precision was 0.732, recall rate was 0.702, and kappa was 0.608.

Clinicopathologic features according to TNBC subtypes

The clinicopathologic features according to Vanderbilt 4 subtypes and surrogate subtypes are compared in Tables 2 and 3. The mean age of patients with the LAR subtype was significantly older than that of patients with other subtypes. In both classifications, patients with the LAR subtype also had significantly more grade 2 tumors, compared with patients who had the other subtypes. Patients with invasive lobular carcinomas all had the LAR subtype in both classifications, whereas metaplastic carcinomas were mainly present in patients with the M subtype, followed by patients with the LAR subtype. In the Vanderbilt 4 classification, the BL subtype tumor size was significantly larger than tumors of other subtypes. In the surrogate subtype classification, BL subtype tumors were significantly larger, as were unclassified tumors. Axillary lymph node status did not differ in either subtype classification. Low-Ki67 tumors were identified more frequently in patients with the LAR subtype, followed by the M subtype, in both classifications.

Table 2
Clinicopathologic features according to Vanderbilt 4 subtypes

		LAR (n = 22), n (%)	IM (n = 32), n (%)	M (n = 38), n (%)	BL (n = 25), n (%)	Unclassified (n = 28), n (%)	p-value
Age	Mean (SD)	61.7 (10.4)	49.7 (15.5)	53.1 (12.7)	53.2 (11.4)	55.6 (11.5)	0.016
	< 35	3 (13.6)	6 (18.8)	7 (18.4)	1 (4.0)	2 (7.1)	0.183
	35–50	17 (77.3)	19 (59.4)	20 (52.6)	13 (52.0)	18 (64.3)	
	> 50	2 (9.1)	7 (21.9)	11 (28.9)	11 (44.0)	8 (28.6)	
Breast Op	BCS	12 (54.5)	29 (90.6)	30 (78.9)	19 (76.0)	21 (75)	0.051
	Mastectomy	10 (45.5)	3 (9.4)	8 (21.1)	6 (24.0)	7 (25)	
Axilla Op	SLNB	14 (63.6)	18 (56.3)	26 (68.4)	16 (64.0)	18 (64.3)	0.948
	ALND	8 (36.4)	13 (40.6)	12 (31.6)	9 (36.0)	10 (35.7)	
	Not done	0 (0)	1 (3.1)	0 (0)	0 (0)	0 (0)	
Histologic subtype	IDCa	13 (59.1)	23 (71.9)	29 (76.3)	21 (84)	25 (89.3)	< 0.001
	ILCa	4 (18.2)	0 (0)	0 (0)	0 (0)	0 (0)	
	Metaplastic	4 (18.2)	1 (3.1)	8 (21.1)	1 (4.0)	2 (7.1)	
	Medullary	0 (0)	7 (21.9)	0 (0)	1 (4.0)	1 (3.6)	
	Others	1 (4.5)	1 (3.1)	1 (2.6)	2 (8.0)	0 (0)	
HG	Gr 2	9 (40.9)	3 (9.4)	5 (13.2)	2 (8.0)	4 (14.3)	0.03
	Gr 3	13 (59.1)	29 (90.6)	33 (86.8)	23 (92.0)	24 (85.7)	

LAR, luminal androgen receptor; IM, Immunomodulatory; M, mesenchymal-like; BL, basal-like; SD, standard deviation; BCS, breast-conserving surgery; SLNB, sentinel lymph node biopsy; ALND, axillary lymph node dissection; IDCa, invasive ductal carcinoma; ILCa, invasive lobular carcinoma; HG, histology grade; Gr, grade; LVI, lymphovascular invasion

* Cancer staging was performed in accordance with the AJCC 7th staging system.

† Ki67 median value at Seoul St. Mary's Hospital is 25%

‡ p-value when excluding LAR subtype

		LAR (n = 22), n (%)	IM (n = 32), n (%)	M (n = 38), n (%)	BL (n = 25), n (%)	Unclassified (n = 28), n (%)	p-value
LVI	No	13 (59.1)	20 (62.5)	26 (68.4)	14 (56.0)	16 (57.1)	0.848
	Yes	9 (40.9)	12 (37.5)	12 (31.6)	11 (44.0)	12 (42.9)	
Tumor size	≤ 2 cm	10 (45.5)	18 (56.3)	13 (34.2)	5 (20.0)	14 (50)	0.048
	> 2, ≤ 5 cm	12 (54.5)	12 (37.5)	24 (63.2)	20 (80.0)	13 (46.4)	
	> 5 cm	0 (0)	2 (6.3)	1 (2.6)	0 (0)	1 (3.6)	
N stage	N0	15 (68.2)	21 (65.6)	27 (71.1)	16 (64.0)	18 (64.3)	0.533
	N1	7 (31.8)	7 (21.9)	10 (26.3)	5 (20.0)	6 (21.4)	
	N2–3	0 (0)	4 (12.5)	1 (2.6)	4 (16.0)	3 (10.7)	
Stage*	I	7 (31.8)	13 (40.6)	13 (34.2)	4 (16.0)	10 (35.7)	0.236
	II	15 (68.2)	16 (50.0)	24 (63.2)	17 (68.0)	15 (53.6)	
	III	0 (0)	3 (9.4)	1 (2.6)	4 (16.0)	3 (10.7)	
Ki67 [†]	< 25%	6 (27.3)	1 (3.1)	4 (10.5)	0 (0)	0 (0)	0.002
	≥ 25%	16 (72.7)	31 (96.9)	34 (89.5)	25 (100)	28 (100)	
AR	Negative	0 (0)	10 (31.3)	14 (36.8)	14 (56.0)	9 (32.1)	< 0.001
	Positive	22 (100)	22 (68.8)	22 (57.9)	11 (44.0)	16 (57.1)	(0.275 [‡])

LAR, luminal androgen receptor; IM, Immunomodulatory; M, mesenchymal-like; BL, basal-like; SD, standard deviation; BCS, breast-conserving surgery; SLNB, sentinel lymph node biopsy; ALND, axillary lymph node dissection; IDCa, invasive ductal carcinoma; ILCa, invasive lobular carcinoma; HG, histology grade; Gr, grade; LVI, lymphovascular invasion

* Cancer staging was performed in accordance with the AJCC 7th staging system.

[†] Ki67 median value at Seoul St. Mary's Hospital is 25%

[‡] p-value when excluding LAR subtype

		LAR (n = 22), n (%)	IM (n = 32), n (%)	M (n = 38), n (%)	BL (n = 25), n (%)	Unclassified (n = 28), n (%)	p-value
CD8	0–10%	7 (31.8)	3 (9.4)	16 (42.1)	5 (20.0)	3 (10.7)	< 0.001
	11–20%	8 (36.4)	3 (9.4)	13 (34.2)	5 (20.0)	7 (25)	
	20–30%	4 (18.2)	11 (34.4)	3 (7.9)	11 (44.0)	7 (25)	
	> 30%	1 (4.5)	15 (46.9)	6 (15.8)	4 (16.0)	9 (32.1)	
CK5/6	Negative	14 (63.6)	11 (34.4)	6 (15.8)	8 (32.0)	13 (46.4)	0.001
	Weak	6 (27.3)	10 (31.3)	15 (39.5)	8 (32.0)	7 (25)	
	Strong	0 (0)	10 (31.3)	16 (42.1)	8 (32.0)	4 (14.3)	
EGFR	Negative	10 (45.5)	6 (18.8)	5 (13.2)	4 (16.0)	4 (14.3)	0.022
	Weak	7 (31.8)	14 (43.8)	18 (47.4)	15 (60.0)	17 (60.7)	
	Strong	2 (9.1)	11 (34.4)	13 (34.2)	5 (20.0)	3 (10.7)	
MUC1	0%	0 (0)	0 (0)	5 (13.2)	0 (0)	0 (0)	0.08
	1–10%	2 (9.1)	3 (9.4)	8 (21.1)	3 (12.0)	3 (10.7)	
	10–50%	1 (4.5)	5 (15.6)	8 (21.1)	6 (24.0)	6 (21.4)	
	> 50%	18 (81.8)	23 (71.9)	17 (44.7)	15 (60.0)	17 (60.7)	
PD-L1	Negative	13 (59.1)	2 (6.3)	25 (65.8)	5 (20.0)	6 (21.4)	< 0.001

LAR, luminal androgen receptor; IM, Immunomodulatory; M, mesenchymal-like; BL, basal-like; SD, standard deviation; BCS, breast-conserving surgery; SLNB, sentinel lymph node biopsy; ALND, axillary lymph node dissection; IDCa, invasive ductal carcinoma; ILCa, invasive lobular carcinoma; HG, histology grade; Gr, grade; LVI, lymphovascular invasion

* Cancer staging was performed in accordance with the AJCC 7th staging system.

† Ki67 median value at Seoul St. Mary's Hospital is 25%

‡ p-value when excluding LAR subtype

		LAR (n = 22), n (%)	IM (n = 32), n (%)	M (n = 38), n (%)	BL (n = 25), n (%)	Unclassified (n = 28), n (%)	p-value
	Positive	9 (40.9)	30 (93.8)	13 (34.2)	20 (80)	19 (67.9)	
p16	Negative	2 (9.1)	6 (18.8)	7 (18.4)	2 (8)	3 (10.7)	0.017
	Mosaic	12 (54.5)	4 (12.5)	16 (42.1)	5 (20)	10 (35.7)	
	Diffuse	8 (36.4)	22 (68.8)	15 (39.5)	18 (72)	14 (50)	
SMAD4	Negative	4 (18.2)	0 (0)	0 (0)	1 (4.0)	1 (3.6)	< 0.001
	Weak	18 (81.8)	10 (31.3)	15 (39.5)	6 (24.0)	12 (42.9)	
	Strong	0 (0)	21 (65.6)	23 (60.5)	17 (68.0)	13 (46.4)	
TP53	0%	14 (63.6)	11 (34.4)	6 (15.8)	8 (32.0)	13 (46.4)	< 0.001
	1–50%	6 (27.3)	10 (31.3)	15 (39.5)	8 (32.0)	7 (25)	
	> 50%	0 (0)	10 (31.3)	16 (42.1)	8 (32.0)	4 (14.3)	
TIL	0–15%	13 (59.1)	2 (6.3)	30 (78.9)	7 (28.0)	6 (21.4)	< 0.001
	15–45%	4 (18.2)	4 (12.5)	2 (5.3)	8 (32.0)	7 (25)	
	45–75%	4 (18.2)	12 (37.5)	4 (10.5)	8 (32.0)	4 (14.3)	
	75–100%	0 (0)	13 (40.6)	0 (0)	1 (4.0)	9 (32.1)	
LAR, luminal androgen receptor; IM, Immunomodulatory; M, mesenchymal-like; BL, basal-like; SD, standard deviation; BCS, breast-conserving surgery; SLNB, sentinel lymph node biopsy; ALND, axillary lymph node dissection; IDCa, invasive ductal carcinoma; ILCa, invasive lobular carcinoma; HG, histology grade; Gr, grade; LVI, lymphovascular invasion							
* Cancer staging was performed in accordance with the AJCC 7th staging system.							
† Ki67 median value at Seoul St. Mary's Hospital is 25%							
‡ p-value when excluding LAR subtype							

Table 3
Clinicopathologic features according to the TNBC surrogate subtype classification

		LAR (n = 26), n (%)	IM (n = 21), n (%)	M (n = 44), n (%)	BL (n = 27), n (%)	Unclassified (n = 18), n (%)	p-value
Age	Mean (SD)	61.3 (12.3)	50.1 (13.2)	54.4 (12.3)	51.7 (12.1)	50.8 (12.2)	0.012
	< 35	5 (19.2)	2 (9.5)	5 (11.4)	2 (7.4)	2 (11.1)	0.292
	35–50	19 (73.1)	11 (52.4)	27 (61.4)	16 (59.3)	9 (50.0)	
	> 50	2 (7.7)	8 (38.1)	12 (27.3)	9 (33.3)	7 (38.9)	
Breast Op	BCS	17 (65.4)	19 (90.5)	33 (75.0)	21 (77.8)	14 (77.8)	0.388
	Mastectomy	9 (34.6)	2 (9.5)	11 (25.0)	6 (22.2)	4 (22.2)	
Axilla Op	SLNB	17 (65.4)	11 (52.4)	30 (68.2)	16 (59.3)	11 (61.1)	0.706
	ALND	8 (30.8)	10 (47.6)	14 (31.8)	11 (40.7)	7 (38.9)	
	Not done	1 (3.8)	0 (0)	0 (0)	0 (0)	0 (0)	
Histologic subtype	IDCa	17 (65.4)	15 (71.4)	33 (75.0)	24 (88.9)	16 (88.9)	0.014
	ILCa	4 (15.4)	0 (0)	0 (0)	0 (0)	0 (0)	
	Metaplastic	3 (11.5)	0 (0)	7 (15.9)	2 (7.4)	1 (5.6)	
	Medullary	1 (3.8)	5 (23.8)	1 (2.3)	1 (3.7)	1 (5.6)	
	Others	1 (3.8)	1 (4.8)	3 (6.8)	0 (0)	0 (0)	
HG	Gr 2	10 (38.5)	1 (4.8)	6 (13.6)	1 (3.7)	3 (16.7)	0.006

LAR, luminal androgen receptor; IM, Immunomodulatory; M, mesenchymal-like; BL, basal-like; SD, standard deviation; BCS, breast-conserving surgery; SLNB, sentinel lymph node biopsy; ALND, axillary lymph node dissection; IDCa, invasive ductal carcinoma; ILCa, invasive lobular carcinoma; HG, histology grade; Gr, grade; LVI, lymphovascular invasion

* Cancer staging was performed in accordance with the AJCC 7th staging system.

† Ki67 median value at Seoul St. Mary's Hospital is 25%

‡ p-value when excluding LAR subtype

		LAR (n = 26), n (%)	IM (n = 21), n (%)	M (n = 44), n (%)	BL (n = 27), n (%)	Unclassified (n = 18), n (%)	p-value
	Gr 3	16 (61.5)	20 (95.2)	38 (86.4)	26 (96.3)	15 (83.3)	
LVI	No	17 (65.4)	13 (61.9)	25 (56.8)	17 (63.0)	11 (61.1)	0.965
	Yes	9 (34.6)	8 (38.1)	19 (43.2)	10 (37)	7 (38.9)	
Tumor size	≤ 2 cm	12 (46.2)	15 (71.4)	18 (40.9)	8 (29.6)	3 (16.7)	0.018
	> 2, ≤ 5 cm	13 (50.0)	6 (28.6)	25 (56.8)	18 (66.7)	14 (77.8)	
	> 5 cm	1 (3.8)	0 (0)	1 (2.3)	1 (3.7)	1 (5.6)	
N stage	N0	17 (65.4)	13 (61.9)	33 (75.0)	16 (59.3)	11 (61.1)	0.455
	N1	7 (26.9)	7 (33.3)	9 (20.5)	8 (29.6)	3 (16.7)	
	N2–3	1 (3.8)	1 (4.8)	2 (4.5)	3 (11.1)	4 (22.2)	
Stage*	I	9 (34.6)	9 (42.9)	17 (38.6)	6 (22.2)	3 (16.7)	0.288
	II	16 (61.5)	11 (52.4)	25 (56.8)	19 (70.4)	11 (61.1)	
	III	1 (3.8)	1 (4.8)	2 (4.5)	2 (7.4)	4 (22.2)	
Ki67 [†]	< 25%	7 (26.9)	0 (0)	2 (4.5)	0 (0)	2 (11.1)	0.002
	≥ 25%	19 (73.1)	21 (100)	42 (95.5)	27 (100)	16 (88.9)	
AR	Negative	0 (0)	9 (42.9)	19 (43.2)	12 (44.4)	6 (33.3)	< 0.001
	Positive	26 (100)	12 (57.1)	25 (56.8)	15 (55.6)	12 (66.7)	(0.884 [‡])

LAR, luminal androgen receptor; IM, Immunomodulatory; M, mesenchymal-like; BL, basal-like; SD, standard deviation; BCS, breast-conserving surgery; SLNB, sentinel lymph node biopsy; ALND, axillary lymph node dissection; IDCa, invasive ductal carcinoma; ILCa, invasive lobular carcinoma; HG, histology grade; Gr, grade; LVI, lymphovascular invasion

* Cancer staging was performed in accordance with the AJCC 7th staging system.

[†] Ki67 median value at Seoul St. Mary's Hospital is 25%

[‡] p-value when excluding LAR subtype

		LAR (n = 26), n (%)	IM (n = 21), n (%)	M (n = 44), n (%)	BL (n = 27), n (%)	Unclassified (n = 18), n (%)	p-value
CD8	0–10%	6 (23.1)	0 (0)	22 (50)	3 (11.1)	1 (5.6)	< 0.001
	11–20%	8 (30.8)	2 (9.5)	16 (36.4)	5 (18.5)	2 (11.1)	
	20–30%	7 (26.9)	2 (9.5)	4 (9.1)	12 (44.4)	10 (55.6)	
	> 30%	3 (11.5)	17 (81.0)	2 (4.5)	7 (25.9)	5 (27.8)	
CK5/6	Negative	16 (61.5)	10 (47.6)	11 (25.0)	7 (25.9)	6 (33.3)	0.003
	Weak	7 (26.9)	7 (33.3)	16 (36.4)	11 (40.7)	4 (22.2)	
	Strong	0 (0)	4 (19.0)	15 (34.1)	9 (33.3)	8 (44.4)	
EGFR	Negative	12 (46.2)	5 (23.8)	8 (18.2)	3 (11.1)	1 (5.6)	0.05
	Weak	9 (34.6)	12 (57.1)	22 (50.0)	17 (63.0)	8 (44.4)	
	Strong	4 (15.4)	4 (19)	9 (20.5)	7 (25.9)	8 (44.4)	
MUC1	0%	0 (0)	0 (0)	3 (6.8)	0 (0)	0 (0)	0.602
	1–10%	2 (7.7)	1 (4.8)	9 (20.5)	3 (11.1)	3 (16.7)	
	10–50%	4 (15.4)	3 (14.3)	7 (15.9)	7 (25.9)	4 (22.2)	
	> 50%	19 (73.1)	17 (81)	25 (56.8)	17 (63)	11 (61.1)	
PD-L1	Negative	13 (50)	0 (0)	33 (75)	3 (11.1)	0 (0)	< 0.001
	Positive	13 (50)	21 (100)	11 ()	24 (88.9)	18 (100)	

LAR, luminal androgen receptor; IM, Immunomodulatory; M, mesenchymal-like; BL, basal-like; SD, standard deviation; BCS, breast-conserving surgery; SLNB, sentinel lymph node biopsy; ALND, axillary lymph node dissection; IDCa, invasive ductal carcinoma; ILCa, invasive lobular carcinoma; HG, histology grade; Gr, grade; LVI, lymphovascular invasion

* Cancer staging was performed in accordance with the AJCC 7th staging system.

† Ki67 median value at Seoul St. Mary's Hospital is 25%

‡ p-value when excluding LAR subtype

		LAR (n = 26), n (%)	IM (n = 21), n (%)	M (n = 44), n (%)	BL (n = 27), n (%)	Unclassified (n = 18), n (%)	p-value
p16	Negative	1 (3.8)	4 (19)	6 (13.6)	0 (0.0)	8 (44.4)	< 0.001
	Mosaic	16 (61.5)	2 (9.5)	16 (36.4)	0 (0.0)	10 (55.6)	
	Diffuse	9 (34.6)	15 (71.4)	22 (50.0)	27 (100)	0 (0.0)	
SMAD4	Negative	1 (3.8)	0 (0)	3 (6.8)	2 (7.4)	0 (0)	0.01
	Weak	19 (73.1)	6 (28.6)	19 (43.2)	7 (25.9)	8 (44.4)	
	Strong	6 (23.1)	15 (71.4)	22 (50.0)	18 (66.7)	10 (55.6)	
TP53	0%	16 (61.5)	10 (47.6)	11 (25.0)	7 (25.9)	6 (33.3)	0.002
	1–50%	7 (26.9)	7 (33.3)	16 (36.4)	11 (40.7)	4 (22.2)	
	> 50%	0 (0)	4 (19.0)	15 (34.1)	9 (33.3)	8 (44.4)	
TIL	0–15%	12 (46.2)	0 (0)	44 (100)	0 (0)	0 (0)	< 0.001
	15–45%	4 (15.4)	0 (0)	0 (0)	13 (48.1)	8 (44.4)	
	45–75%	6 (23.1)	2 (9.5)	0 (0)	14 (51.9)	10 (55.6)	
	75–100%	3 (11.5)	19 (90.5)	0 (0)	0 (0)	0 (0)	
LAR, luminal androgen receptor; IM, Immunomodulatory; M, mesenchymal-like; BL, basal-like; SD, standard deviation; BCS, breast-conserving surgery; SLNB, sentinel lymph node biopsy; ALND, axillary lymph node dissection; IDCa, invasive ductal carcinoma; ILCa, invasive lobular carcinoma; HG, histology grade; Gr, grade; LVI, lymphovascular invasion							
* Cancer staging was performed in accordance with the AJCC 7th staging system.							
† Ki67 median value at Seoul St. Mary's Hospital is 25%							
‡ p-value when excluding LAR subtype							

Similar characteristics related to the IHC results of biomarkers not included in the surrogate subtype classification were also identified in both classifications (Tables 2 and 3). A high PD-L1-positive rate was identified among patients with the IM and BL1 subtypes. Notably, all IM tumors from the surrogate

classification exhibited with PD-L1-positive staining. EGFR and CK5/6 showed negative or weak expression among patients with the LAR subtype. Whereas EGFR and CK5/6 did not show any specific pattern among patients with the BL subtype, compared with patients who had the IM or M subtypes. SMAD4 and TP53 also showed negative or weak expression among patients with the LAR subtype.

The median follow-up duration was 41 months (range, 0–64 months). During the study period, 25 patients (17.2%) experienced locoregional recurrence or distant metastasis. Disease-free survival did not differ according to the Vanderbilt 4 subtypes, but a tendency for worse survival was noted among patients with the M subtype ($p = 0.16$, Fig. 4). When classified according to surrogate subtypes, patients with the M subtype demonstrated significantly worse disease-free survival ($p = 0.004$, Fig. 2). M subtype persisted as an independent risk factor for poor survival after adjusting for other prognostic factors (HR 11.401; 95% CI 1.488–87.370; $p = 0.019$; Table 4). Although it was not statistically significant, a tendency for better survival among patients with the IM subtype was also identified in both classifications. No difference in overall survival was noted in either classification (Vanderbilt 4 subtypes overall survival, $p = 0.238$; surrogate subtypes overall survival, $p = 0.061$; Fig. 4).

Table 4

Univariate and multivariate analysis of prognostic factors and TNBC surrogate subtype classification

		Univariate				Multivariate			
		HR	95% CI		<i>p</i> -value	HR	95% CI		<i>p</i> -value
Age	continuous	1.046	1.010	1.083	0.011	1.061	1.022	1.104	0.002
HG	Gr 3 vs Gr 2	0.984	0.338	2.869	0.977				
LVI	Yes vs No	1.574	0.718	3.455	0.258				
Tumor size	continuous	1.201	0.933	1.547	0.156				
N stage	N(+) vs N0	2.455	1.120	5.381	0.026	2.632	1.170	5.924	0.019
Ki67 (%)	continuous	1.833	0.248	13.570	0.553				
TNBC surrogate subtype	IM	Reference				Reference			
	LAR	4.029	0.450	36.100	0.213	2.716	0.297	24.863	0.376
	M	10.100	1.331	76.670	0.025	11.401	1.488	87.370	0.019
	BL	2.969	0.309	28.560	0.346	3.315	0.343	32.049	0.301
	Unclassified	1.231	0.077	19.670	0.883	1.367	0.085	21.921	0.825
HG, histology grade; Gr, grade; LVI, lymphovascular invasion; LAR, luminal androgen receptor; IM, Immunomodulatory; M, mesenchymal-like; BL, basal-like									

Discussion

TNBC is a heterogeneous disease and subtype classification is necessary for optimization of personalized treatment. TNBC subtyping using gene expression data has been implemented by several groups but gene expression data are impractical and expensive for clinical application. To aid in this subtyping effort, we developed a TNBC surrogate subtype classification to classify TNBC into four subtypes that correlate with the Vanderbilt subtypes. The TNBC surrogate subtyping adopts p16, AR, and TIL data for classification, which are widely used parameters in pathology clinics.

This is the first study to develop a TNBC surrogate subtype that correlates with the Vanderbilt subtypes. Zhao et al. developed an IHC-based approach for TNBC subtyping by comparison with mRNA-based subtypes used in the Fudan University Shanghai Cancer Center [26]. Other similar studies have attempted to classify TNBC subtypes by IHC panels but have focused on independent classifications that did not involve correlations with any gene expression data [13, 16].

The LAR subtype is universally identified among TNBC gene expression subtypes that use unsupervised clustering methods [4–7]. Notably, the LAR subtype is enriched in AR expression and its downstream gene targets and is relatively simple to identify using AR protein expression in IHC analysis [4, 27, 28]. In this study, we used an AR Allred score of 8 to identify the LAR subtype. This comprised 18% of the TNBC cohort, similar to previous reports in which 10–35% of TNBC tumors expressed AR [29–32]. The clinical significance of AR expression has been identified in several clinical trials, indicating the efficacy of anti-androgen treatment in patients with AR-positive metastatic TNBC [33, 34]. Clinical trials of anti-androgens have used cutoff values of 1% or 10%, which differ from our study. The predictive value of AR expression levels for anti-androgen treatment requires further investigation, but the present study implies that a higher cutoff value may be needed to identify a LAR subtype more specifically in patients with TNBC.

Another subtype consistently identified in distinct gene expression classification systems is the IM subtype [4, 6, 7, 11]. Gene expression data have shown that this subtype is highly enriched for immune cell signaling, and that it has a high prevalence of TILs [10, 11]. This correlates with the use of TIL as a marker to determine IM subtype in the surrogate subtype classification. The clinical implications of the IM subtype and TIL as a subtype marker are related to the use of immune checkpoint inhibitor therapies, which are currently a novel component of care for patients with TNBC [35]. Atezolizumab, a PD-L1 inhibitor, has been approved in combination with nab-paclitaxel for PD-L1-positive metastatic TNBCs and TIL is a predictive factor when tumors are PD-L1 positive [36, 37]. The predictive value of TILs has also been identified in clinical trials for pembrolizumab, a PD-1 inhibitor [38, 39]. In this study, both IM subtypes classified on the basis of RNA and IHC expression exhibited high CD8 + levels and high rates of PD-L1 positivity, which are additional biomarkers that serve as predictive factors for immune checkpoint inhibitors [40].

In contrast to the IM subtype, low TIL (< 20%) was the determining factor for the M subtype in the IHC classification. Lehmann et al. reported that M and IM subtypes had a negative correlation, which was consistent with the classification method in our study [10]. The negative correlation between the M and IM subtypes implies that the M subtype involves an immunosuppressed microenvironment, corresponding to the poor prognosis of patients with the M subtype [10]. The clinical significance of the M subtype as a target for novel therapeutics is unclear. Bioinformatics analysis of data derived from the Molecular

Taxonomy of Breast Cancer International Consortium (METABRIC) and The Cancer Genome Atlas (TCGA) showed that the M subtype is associated with an elevated angiogenesis signature score and enrichment of the EGFR and Notch signaling pathways [17]. On the basis of these results, antiangiogenic therapy or EGFR inhibitors could be considered for patients with M subtype tumors, which have both failed to demonstrate a survival benefit in unselected TNBC populations [41, 42].

Immunostainings for basal cytokeratins including CK5/6, CK14, CK17, and EGFR have been regarded as surrogate markers of the BL subtype. However diffuse and strong staining of p16 immunostaining is the last condition of BL subtype in our TNBC surrogate subtype classification. This correlates with the TNBC cohort from the METABRIC and TCGA dataset, showing copy number amplifications in *CDKN2A/B* genes in the BL1 subtype [17]. p16 regulates the cell cycle by inhibition of phosphorylation of Rb via inactivation of CDK4/6 [43]. The positive immunostaining of p16 is suggested as a surrogate marker for Rb pathway loss, and Rb1 loss is associated with homologous recombination deficiency in high-grade serous ovarian cancer [45, 46]. This suggests that adopting p16 immunostaining as a surrogate marker for BL subtype in our TNBC surrogate subtype classification is related to Rb1 loss and homologous recombination deficiency. The BL subtype itself is characterized by high genomic instability and high copy number losses for BRCA1/2, which implies sensitivity to poly-ADP ribose polymerase(PARP) inhibitors. PARP inhibitors have shown considerable benefit in patients with advanced breast cancer and a germline BRCA1/2 mutation, especially those with TNBC tumors [48, 49]. Currently, the presence of germline BRCA 1/2 mutations is the main predictive factor for the effects of PARP inhibitors, but the BL subtype could also be regarded as a predictive factor, especially in patients with wild-type BRCA 1/2 with homologous recombination deficiency.

The strength of the TNBC surrogate subtype classification is that AR, p16 IHC, and TIL evaluation protocols are widely used in pathology clinics. Guidelines for IHC procedures and TIL assessment are already established [20, 24], so the clinical application of the surrogate subtype classification is easy and immediately available.

However, there were some weaknesses in this study. Tissue microarrays were used for TIL evaluation and IHC staining, which may not represent the entire tumor. RNA sequencing was performed using bulk tumor tissue, which does not reflect the intratumoral heterogeneity demonstrated by single-cell RNA sequencing [50]. Eighteen patients (13.2%) were unclassified in the surrogate subtype classification and could not be included in any specific Vanderbilt 4 subtype. This lack of classification requires further investigation. CART is a relatively unstable method, whereby a small change in the data can cause considerable variation in the model, especially when a small sample size is used [51]. The TNBC cohort in this study was relatively small and further validation is needed using a larger, external dataset. Also, all patients included in this study underwent primary surgery followed by adjuvant chemotherapy, which is deviated from current treatment guidelines that recommend preoperative systemic therapy in patients with TNBC tumors. An additional study using core needle biopsy tissues in patients undergoing neoadjuvant therapy is needed to confirm the application of the surrogate subtypes in current TNBC treatment.

Conclusions

In this study, we developed a novel TNBC surrogate subtype classification that correlates with the gene expression classification established by the Vanderbilt group. The surrogate subtype adopts AR, TIL, and p16, thereby classifying TNBC with a practical and easily accessible diagnostic test. Each subtype exhibits diverse clinicopathological and prognostic characteristics. Further studies are needed to validate the clinical utility of the surrogate subtype classification for use in personalized treatment with targeted and immune-based therapies.

Abbreviations

AR

Androgen receptor

BL

Basal-like

CART

Classification and regression tree

FFPE

Formalin-fixed paraffine embedded

IHC

Immunohistochemical

IM

Immunomodulatory

LAR

Luminal androgen receptor

M

Mesenchymal-like

METABRIC

Molecular Taxonomy of Breast Cancer International Consortium

MSL

Mesenchymal stem-like

PARP

poly-ADP ribose polymerase

TCGA

The Cancer Genome Atlas

TIL

tumor-infiltrating lymphocytes

TNBC

Triple-negative breast cancer

Declarations

Ethics approval and consent to participate

This study was approved by the institutional review board of Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea (IRB no. KC17TNSI0414), and the requirement for informed consent was waived.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author Contributions

T.-K.Y and B.-J.C performed study concept and design; T.-K.Y. and J.K. performed development of methodology and writing, review and revision of the paper; T.-K.Y., J.K., A.L. and B.-J.C. provided acquisition, analysis and interpretation of data, and statistical analysis. All authors read and approved the final paper.

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Figures

Figure 1

Representative images of TNBC surrogate classification. Hematoxylin and eosin (H&E) stain (A-E), tumor-infiltrating lymphocytes (TIL); A:20, B:90, C:10, D:20, E: 60. Androgen receptor immunohistochemical stain (F-J); Allred score F:8, G:0, H:1, I:0, J:1. p16 immunohistochemical stain (K-O); K, N: diffuse and strong, L, M: weak and mosaic, O: negative.

Figure 2

TNBC surrogate subtype classification method

Figure 3

Concurrence between TNBC surrogate subtype classification and gene expression-based classification (Vanderbilt 4 subtypes). (A) Bar chart showing concurrence between TNBC surrogate subtypes and Vanderbilt 4 subtypes. (B) Confusion matrix for concurrence between TNBC surrogate subtypes and Vanderbilt 4 subtypes. SUR, TNBC surrogate subtype classification; UNC, unclassified

Figure 4

Kaplan–Meier curves of disease-free survival and overall survival according to Vanderbilt 4 subtypes and surrogate subtypes

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