

Comparison among Various Prognostic Pathologic Markers Revealed Significantly Poorer Prognosis in Non-basal-like than in Basal-like Triple Negative Breast Cancer

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Abstract

Objective: To find the incidence and prognosis of basal-like (TNB) and non-basal-like (TNN) in triple negative breast cancer. **Methods:** Triple negative breast cancer (TN) cases were retrospectively reviewed and biomarkers studied on tissue microarray for ER, PR, HER2, Ki67, basal cytokeratins (CK5/6, CK14, CK17), EGFR, CD117, p63, p53, vimentin, CK7, CK8/18, CK19, BCL2, p16, WT1, and cyclin D1. The patients were reclassified according to ER, PR, HER2, Ki67 index, basal CKs and EGFR results into: lumA, lumB, HER2+, TNB and TNN. **Results:** From 2007 to 2010, there were 193 female patients (120 TNB, 17 TNN, 42 lumB, and 14 HER2+). There were 184 (95.3%) invasive ductal carcinoma (IDC). All 17 TNN were IDC. High grade histology accounted for 71.5%. The median follow up time was 62.93 months. There were no significant differences in age, histologic grade, and tumor size between TNB and TNN while TNN had significant number of higher stage ($p=0.028$), axillary lymph node metastasis of more than 3 nodes ($p=0.005$) and lower disease free survival (DFS, $p=0.004$) and overall survival (OS, $p=0.001$). TNN also had the poorest prognosis among the four subtypes. Tumor size and axillary nodal involvement were the independent predictors for DFS and OS. Absence of EGFR expression was an independent factor for lower DFS and OS in TN. Absence of CK8/18 expression was an independent factor for lower DFS in any combined group and OS in the combined TN and lumB group. Absence of p16 expression was an independent factor for lower DFS and OS of all cohort. **Conclusion:** TNN accounted for 12.3% of TN and had poorer prognosis. Tumor size and axillary nodal involvement were the independent predictors for DFS and OS. Absence of EGFR, CK8/18 or p16 expression had influence on survival in TN or combined group.

Keywords: Triple negative breast cancer- basal-like- non-basal-like- prognosis

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Introduction

Breast cancer is the most common cancer in most countries, including Thailand, and it has been classified according to gene expression profiling into 5 subtypes [1]. Immunohistochemical (IHC) markers of specific gene expression products were then proposed [1-3] with some modification in the St. Gallen consensus on the primary therapy of early breast cancer [4] to classify

breast cancer as a practical basis for treatment, as follows: luminal A (lumA), luminal B (lumB), HER2+ (HER2+), and triple-negative (TN). TN subtype is subgrouped into [1] basal-like TN (TNB) when it is immunophenotypically negative ER, PR, and HER2 with at least any basal cytokeratin (basal CK; CK5, CK14, CK17) or epidermal growth factor receptor (EGFR)

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expression, and [2] non-basal-like TN (TNN) when it is immunophenotypically negative ER, PR, HER2, basal CK, and EGFR.

Of the 5 subtypes, TN is usually poorly differentiated, has aggressive clinical behavior, and has a poor prognosis. Moreover, therapy for TN subtype is limited due to the lack of hormone receptors, and HER2 expression of the tumor. Several studies have investigated the predictive/prognostic factors of this breast cancer subtype, especially factors that could lead to targeted therapies.

We previously classified 100 Thai breast cancer patients recruited during 2002-2004 according to the results of IHC study and found a prevalence of TNB and TNN of 15% and 14%, respectively [5]. We found no significant difference in age, tumor size, lymphovascular invasion (LVI), nodal metastasis, microvessel density, expression of vascular endothelial growth factor (VEGF), or survival between subtypes. Moreover, we were unable to find significant clinical difference between TNB and TNN due to our small sample size. Comparison between TN and non-TN revealed significantly higher tumor grade, mitotic count expressed by Ki-67 index, p53, and vimentin expression, and decreased overall survival (OS) in TN subtype compared to non-TN subtype [6, 7].

In the current retrospective study of a larger population, we focused on the identification of TNB and TNN using biomarkers, clinical outcomes, and predictive/prognostic factors in the TN subtype.

Materials and Methods

The protocol for this study was approved by the Institutional Review Board of the Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand [COA no. R635/2553(EC3)]. We performed a retrospective study of archival formalin-fixed paraffin-embedded (FFPE) breast cancer specimens previously reported ER-, PR-, and HER2- (IHC 0/1+) or equivocal (IHC 2+) during 2007 to 2010. Cases with adequate tissue for study were enrolled, and the histology and prognostic/predictive factors of the retrieved tumors were reviewed by 3 pathologists, TC, NS and MW.

Construction of tissue microarray (TMA) and IHC study Three cores of 2 mm diameter FFPE tissue from each case and 6 controls (breast cancer with ER+, PR+, HER2+; breast cancer with p53+; fibroadenoma; appendix; lung; placental tissue) were constructed averaging 24 cases per one recipient block. Cases with HER2 IHC2+ were embedded in a separate recipient block. Each block was cut and stained for ER, PR, and HER2, markers of basal-like subtype [three basal CKs (CK5, CK14, and CK17) and EGFR], CD117, p63, p53, vimentin, Ki67, CK7, CK8/18, CK19, BCL2, p16, WT1, and cyclin D1. Dual in situ hybridization (DISH) for *HER2* was performed on the block with *HER2* IHC2+ by using the Ventana *HER2* Dual ISH DNA Probe Cocktail Assay. Details of the antibodies used and the criteria for interpretation are provided in Table 1.

All stained slides were scanned using an Aperio ScanScope (Leica Biosystems, Wetzlar, Germany),

and IHC interpretation was performed manually on the scanned slides by TC and NS who were blinded to the clinical outcomes. The HER2 DISH was interpreted by NS. ER, PR, and *HER2* results were evaluated according to the ASCO/CAP guidelines [8, 9]. Patients were re-classified into 5 groups as follows: 1) lumA (ER+ and/or PR+, HER2-, and Ki-67 $\leq 14\%$), 2) lumB (ER+ and/or PR+, and HER2+ or ER+ and/or PR+, HER2-, and Ki-67 $> 14\%$), 3) HER2+ (ER-, PR-, and HER2+), 4) TNB (ER-, PR-, and HER2- with at least any CK5+, CK14+, CK17+, or EGFR+), and 5) TNN (ER-, PR-, HER2-, CK5-, CK14-, CK17-, and EGFR-).

Of 1,561 breast cancer patients (1,163 cases with predictive factor study) who underwent mastectomy or wide excision at Siriraj Hospital during 2007 to 2010, there were 276 patients with ER-, PR-, or HER2- (score 0/1+) or equivocal (score 2+) documented in the pathology reports. After review, 203 patients with adequate tissue for study were proceeded for TMA construction and IHC staining. A small number of cases with ER+/PR+ and HER2+ were identified. Twenty-four patients with equivocal HER2 status and HER2+ (IHC3+) had *HER2* DISH performed, which revealed positivity in 18 patients (14 patients HER2 IHC3+ on pathology review, and 4 patients with HER2 IHC2+).

Pathological staging parameters, including tumor size, LVI, and nodal status, were obtained from review of the pathology reports. Clinical data, including age, mode of treatment, and survival status (recurrence and metastasis/death), were retrieved from medical records, patient communication, and Thai population database.

Statistical analysis

Descriptive statistics were used to summarize demographic data, clinical data, and Ki67 index. Chi-square test was used to compare biomarker expressions between TNB and TNN, and between TN and non-TN (lumB and HER2). P-values were adjusted for multiple comparisons using a modified Bonferroni correction method.

For survival analysis, the follow-up period was defined as the time from the operation date to the date of the last visit/observation or death. Disease-free survival (DFS) was defined as the time between the date of the operation and the date of relapse (recurrence/metastasis). Overall survival (OS) was defined as the time between the date of the operation and the date of death. Log-rank test was used to estimate and compare survival between groups. A p-value of < 0.05 was considered statistically significant.

Results

According to the IHC results of ER, PR, HER2, basal CK, and EGFR, there were 121 TNB, 17 TNN, 42 lumB, and 14 HER2+ patients with complete follow-up data. A total of 193 female patients were clinicopathologically assessed. The clinical, histologic, and IHC studies are summarized in Tables 2 and 3.

Table 1. Antibodies Used and Positive Criteria

Antibody	Clone	Source	Dilution	Positive criteria
ER	SP1	Ventana	prediluted	≥1% nuclear staining
PR	1E2	Ventana	prediluted	≥1% nuclear staining
HER2	4B5	Ventana	prediluted	Score 3+, strong, complete membrane staining in >10% of invasive tumor cells
CK5/6	D5&16B4	Cell Marque	1:100	≥1% cytoplasmic and membrane staining
CK14*	LL002	Novocastra	1:200	≥1% cytoplasmic and membrane staining
CK17*	E3	Novocastra	1:100	≥1% cytoplasmic and membrane staining
EGFR	5B7	Ventana	prediluted	≥1% membrane staining
CD117	C-kit	DAKO	1:1000	≥10% membrane staining
p63	4A4	DAKO	prediluted	Any nuclear staining
p53	D0-7	Cell Marque	1:500	≥10% nuclear staining
Vimentin	V9	DAKO	1:500	At least 10% cytoplasmic staining
Ki-67	MIB1	DAKO	1:300	Counted from at least 200 tumor cells, expressed in%
CK7	OV-TL12/30	Cell Marque	1:1000	≥1% cytoplasmic and/or membrane staining; score#
CK8/18	SP3	Thermo Scientific	1:100	≥1% cytoplasmic and/or membrane staining; score#
CK19	A53-B/A2.26	Thermo Scientific	1:300	≥1% cytoplasmic and/or membrane staining; score#
BCL2	124	DAKO	1:100	≥1% cytoplasmic membrane staining; score#
p16	E6H4	Ventana	prediluted	Cytoplasmic and nuclear staining; score#
WT1	6F-H2	Cell Marque	1:500	Any nuclear staining Cytoplasmic staining, score#
Cyclin D1	SP4	Thermo Scientific	1:100	Any nuclear staining; score#

All were performed using an automated immunostainer. *Performed from the Institute of Pathology, Ministry of Public Health, Thailand; #score=(intensity,1-3)+(quantity,1-5); intensity, 1=mild, 2=intermediate, 3=strong; quantity, 0=no positive cell, 1=<1, 2=≥1-10, 3=>10-33, 4=>33-66, 5=>66-100% positive cells

Overall characteristics of included cases (Table 2)

The mean age at diagnosis was 52.3±13.1 years (range: 26-88 years, median: 51 years). There were 184 (95.3%) invasive ductal carcinomas, not otherwise specified (IDC, NOS), 1 invasive lobular (ILC), 5 metaplastic, 1 adenosquamous, 1 medullary-like, and 1 mucinous carcinoma with micropapillary-like feature. High-grade tumor accounted for 71.5%. Tumor size ranged from 0.5 to 19 cm (mean: 3.22±2.4 cm). Modified radical mastectomy was performed in 89 (46.1%) patients. Total mastectomy with sentinel lymph node (SNL) biopsy with or without axillary node dissection was performed in 59 (30.6%) patients. SLN was performed in 55.4%, and 9.3% were positive. Among 193 patients, 32, 103, 6, and 52 patients had stage 1, 2, 3, and 4 at diagnosis, respectively. Positive axillary nodes were found in 37.8% of patients, of which 50.8% had perinodal invasion. LVI was observed in 37.3% of all patients. Neoadjuvant chemotherapy was given to 11.4% of patients, and 81.9% received postoperative chemotherapy. Less than half of the patients (46.5%) received radiation therapy. The follow-up time ranged from 0.53 to 96.53 months (median follow-up: 62.93 months). Of those, 10.9% and 30.3% had locoregional recurrence and distant metastasis, respectively; 61.7% were alive and disease-free; 31.1% had relapse or metastasis during the course of their disease; and, 34.7% died of their disease.

Associations between clinicopathological parameters and subtypes

Patients with TNN breast cancer had significantly older age than those with lumB breast cancer ($p=0.013$).

No significant difference in tumor size was found among the TNB, TNN, and lumB subtypes. LumB was associated with the lowest proportion of more than 3 axillary node involvement, while TNN was associated with the highest proportion ($p=0.005$), and so did TN when compared with lumB ($p=0.003$) (Table 2). Forty-eight patients had metastasis at the time of diagnosis. TNN had higher stages than TNB or lumB ($p=0.028$), and so did TN versus lumB ($p=0.020$).

One-third of TNN and 8.3% of TNB received neoadjuvant treatment, whereas no HER2+ received neoadjuvant chemotherapy. Of 174 patients with known history of chemotherapy treatment, 90.8% received neoadjuvant and/or postoperative adjuvant chemotherapy, including 92.5% of TNB, 82.4% of TNN, 86.8% of lumB, and all HER2+. Of those, 81.6%, 78.6%, 84.8%, and 84.6% received anthracyclines either singly or in combination with others, respectively. TN had a significantly higher recurrence rate than non-TN.

Biomarker expressions among different subtypes

IHC of biomarkers according to the 4 reclassified subtypes are summarized in Table 3. ER, PR, and HER2 of lumB were expressed in 40.5%, 76.2%, and 9.5%, respectively.

Table 2. Clinicopathologic Features of Studied Population According to Breast Cancer Subtypes

	TNB no.of cases (120, 62.2%)	TNN no.of cases (17, 8.8%)	LumB no.of cases (42, 21.8 %)	HER2 no.of cases (14, 7.3%)	P (overall TNB vs TNN vs lumB)	P (TN vs lumB)	P TN vs nonTN
Mean age (years)	52.1	59.9	49.12	54.21	0.013 (overall)	0.053	0.199
(95% CI)	(49.8-54.5)	(52.6-67.2)	(45.7-52.5)	(44.98-63.45)	0.013		
(min, max)	(27, 86)	(37, 88)	(32, 80)	(26, 85)	(lumB vs TNN)		
Histologic subtype							
IDC	113 (94.2)	17 (100)	40 (95.2)	14 (100)			
ILC	0	0	1 (2.4)	0			
Metaplastic carcinoma	5 (4.2)	0	0	0			
Adenosquamous carcinoma	1 (0.8)	0	0	0			
Medullary-like features	1 (0.8)	0	0	0			
Mucinous carcinoma	0	0	1 (2.4)	0			
Histologic grade (IDC)							
1	3 (2.6)	0	0	0			
2	32 (28.1)	6 (35.3)	7 (17.5)	3 (21.4)			
3	79 (69.3)	11 (64.7)	33 (82.5)	11 (78.6)	0.381	0.195	0.156
Tumor size, mean							
(95% CI)	(2.73-3.53)	(2.28-7.06)	(2.59-3.68)	(1.92-2.89)			
(min, max)	(0.8-19.0)	(1.2-19.0)	(0.5-10.0)	(1.5-4.0)			
≤2 cm	40 (33.3)	3 (17.6)	14 (33.3)	7 (50)			
2-5 cm	70 (58.3)	11 (64.7)	25 (59.5)	7 (50)			
> 5 cm	10 (8.3)	3 (17.6)	3 (7.1)	0	0.569	0.888	0.53
LVI (+/-)(%+)	40/79 (33.6)	6/11 (35.3)	16/26 (38.1)	9/5 (64.3)	0.871	0.612	0.169
Axillary node status							
Negative	81 (67.5)	9 (52.9)	24 (57.1)	6 (42.9)			
Positive nodes 1-3	17 (14.2)	2 (11.8)	15 (35.7)	4 (28.6)			
≥4	22 (18.3)	6 (36.3)	3 (7.1)	4 (28.6)	0.005	0.003	
Perinodal invasion (+/-) (%+)	18/13 (60)	0/7	10/7 (58.8)	3/4 (42.9)	0.016	0.432	0.602
Staging							
1	26 (21.7)	1 (5.9)	8 (19.0)	4 (28.6)			
2	70 (58.3)	7 (41.2)	28 (66.7)	6 (42.9)			
3	20 (16.7)	6 (35.3)	4 (9.5)	3 (21.4)			
4	4 (3.3)	3 (17.6)	2 (4.8)	1 (7.1)	0.028	0.02	0.758
Staging group							
1+2	96 (80.0)	8 (47.1)	36 (85.7)	10 (71.4)			
3+4	24 (20.0)	9 (52.9)	6 (14.3)	4 (28.6)	0.004	0.002	0.345
Surgery							
Wide excision+SLNB	21 (17.5)	1 (5.4)	3 (7.14)	0			
Wide excision+ALND	16 (13.3)	1 (5.4)	2 (4.8)	1 (7.1)			
TM+SLNB	37 (30.8)	3 (17.6)	7 (16.7)	2 (14.3)			
TM+SLNB+ LD flap	7 (5.8)	1 (5.4)	2 (4.8)	0			
MRM	39 (32.5)	11 (64.7)	28 (66.7)	11 (78.6)			
Neo-adjuvant therapy (total, 174)							
Anthracycline-based	9 (90.0)	3 (50.0)	5 (83.3)				
Anthracycline-based + taxane	0	1 (16.7)	0				
Others	1 (10.0)	2 (33.3)	1 (16.7)				
Chemotherapy*(total, 174)							
Anthracycline-based	66 (67.3)	6 (42.9)	23 (69.7)	8 (61.5)			
Anthracycline-based + taxane	14 (14.3)	5 (35.7)	5 (15.2)	3 (23.1)			
Others	18 (18.4)	3 (21.4)	5 (15.2)	2 (15.4)			
None	8 (7.5)	3 (17.6)	5 (13.2)	0			

Abbreviations: TNB, triple negative basal-like; TNN, triple negative non-basal-like; TN, triple negative breast cancer, TNB&TNN; lumB, luminal B; IDC, invasive ductal carcinoma; LVI, lymphovascular invasion; SLNB, sentinel lymph node biopsy; ALND, axillary lymph node dissection; TM, total mastectomy; LD, latissimus dorsi; MRM, modified radical mastectomy; *, neoadjuvant and postoperative

Continued Table 2.

	TNB no.of cases (120, 62.2%)	TNN no.of cases (17, 8.8%)	LumB no.of cases (42, 21.8 %)	HER2 no.of cases (14, 7.3%)	P (overall TNB vs TNN vs lumB)	P (TN vs lumB)	P (TN vs nonTN)
Radiation	53 (44.2)	9 (52.9)	17 (40.5)	2 (21.4)			
Recurrence (+-)(%+)	32/88 (26.7)	10/7 (58.8)	14/28 (33.3)	5/9 (35.7)	0.26	0.743	0.009
Status							
Alive, disease free	79 (65.8)	5 (29.4)	28 (66.7)	7 (50.0)			
Alive with disease	5 (4.2)	1 (5.9)	2 (4.8)	0 (0.0)			
Dead	36 (30.0)	11 (64.7)	12 (28.6)	7 (50.0)	0.057	0.723	0.754

Abbreviations: TNB, triple negative basal-like; TNN, triple negative non-basal-like; TN, triple negative breast cancer, TNB&TNN; lumB, luminal B; IDC, invasive ductal carcinoma; LVI, lymphovascular invasion; SLNB, sentinel lymph node biopsy; ALND, axillary lymph node dissection; TM, total mastectomy; LD, latissimus dorsi; MRM, modified radical mastectomy; *, neoadjuvant and postoperative

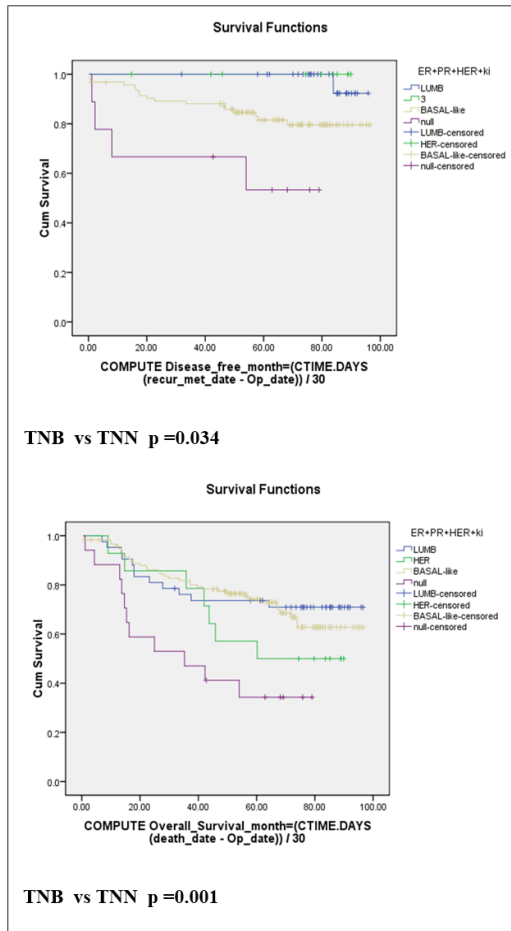


Figure 1. Disease Free Survival (DFS) and Overall Survival (OS) According to Subtypes

Basal-like biomarker expressions

Basal CKs: Both TNB and lumB contained basal CKs (CK 5/6, CK14, or CK17) in a significant number. They were found in 35.7% of HER2+. Among these, CK5/6 had greater sensitivity (70.0%) for detecting basal-like subtype when compared with other CKs. When all three were used, 81.7% of TNB could be diagnosed.

EGFR: This protein was detected in nearly equal proportions in TNB and lumB (74.2% and 71.4%, respectively), and in less than half of HER2+. It was insignificantly more prevalent than CK5/6 (18.3% of TNB and 9.5% of lumB had positive EGFR alone). With the use of CK5/6 and EGFR, 98.3% of TNB could be detected.

CD117: The distribution of CD117 expressions was significantly different with no expression in HER2+, low frequency in TNN (23.5%), and higher frequency in lumB and TNB (26.2% and 45.0%, respectively).

p63: This protein had low sensitivity (15.8% positive) when compared with basal CKs for detecting TNB. In addition, it was not expressed in TNN. It was present in all 5 metaplastic and adenocarcinomas, and in one-third of lumB; however, it declined significantly when TN (TNB and TNN) subtype was compared with lumB (13.9% vs. 38.1%, respectively).

Vimentin: This marker was expressed in only 10% of HER2 (p=0.020) while nearly 30-50% of tumors in the three remaining subtypes.

p53: This protein was expressed in more than 50% of tumors in all subtypes without significant difference in distribution.

Ki-67 index: Prolonged storage of tissue caused diminished staining, so repeat staining with increased concentration was performed. Ki67 index was high in all subtypes with a median value of 45%, except for the HER2+ subtype, which had a significantly lower Ki67 index (30.13%, p=0.005).

CD34: There was no expression of this protein in tumor cells, including spindle cells in metaplastic carcinoma. No stromal cell staining was observed.

CK7: Only 5 patients had no expression of this marker. Expression was similar in all subtypes with approximately 90% positive when using a cutoff score of 3.

CK8/18: Only 3 patients had no CK8/18 expression. Expression among subtypes (score of at least 3) was significantly different with highest expression of 100% in HER2+, and lowest expression of 76.5% in TNN subtypes. Three patients had no CK7 expression, but had CK8/18 expression.

CK19: Six patients had no CK19 expression. One of them had negative CK7 expression, and two had neither CK7 nor CK8/18 expression. All patients with negative CK19 also had negative BCL2. (data not shown in Table 3) **BCL2:** There was no BCL2 expression in 63%, and less than 10% expression in 20.8% of patients. HER2+ subtype had no expression regardless of cutoff score. When using a cutoff score of less than 10%, TNN subtype also had no expression.

p16: There was no expression in 14.6%, and diffuse strong expression in 47.1% of all patients. There was no

Table 3. Biomarker Expressions According to Reclassified Breast Cancer Subtypes

Antibody		Total no. (193, 100%)	TNB no.of cases (120, 62.2%)	TNN no.of cases (17, 8.8%)	LumB no.of cases (42, 21.8%)	HER2 no.of cases (14, 7.3%)	P All subtypes	P (TNB, TNN and lumB)	P (TN vs lumB)	P (TN vs Non-TN)
ER	Negative		120 (100)	17 (100)	25 (59.5)	14 (100)				
	Positive		0	0	17 (40.5)	0				
PR	Negative		120 (100)	17 (100)	10 (23.8)	14 (100)				
	Positive		0	0	32 (76.2)	0				
HER2	Negative		120 (100)	17 (100)	38 (90.5)	0				
	Positive		0	0	4 (9.5)	14(100)				
Ki-67 (95%CI, SD)		45.7 (43.05-48.35, 18.66)	46.9 (43.41-50.38, 19.28)	41.61 (33.72-49.49, 15.34)	49.11 (43.63-54.59, 17.58)	30.13 (23.24-37.02, 11.94)	0.005	0.373	0.394	0.528
Ki-67	≤14	3 (1.6)	2 (1.7)	0	0 (2.4)	1 (7.1)				
	>14-30	41 (21.2)	23 (19.2)	6 (35.3)	5 (11.9)	7 (50.0)				
	>30-50	72 (38.9)	44 (36.7)	5 (29.4)	18 (42.9)	5 (35.7)	0.038	0.524	0.499	0.879
	>50	77 (39.9)	51 (42.5)	6 (35.3)	19 (45.2)	1 (7.1)				
CK5/6	Negative	82 (42.5)	37 (30.8)	17 (100)	16 (38.1)	12 (85.7)				
	Positive	111 (57.5)	83 (69.2)	0	26 (61.9)	2 (14.3)			0.945	0.177
CK14	Negative	110 (57.0)	62 (51.7)	17 (100)	19 (45.2)	12 (85.7)				
	Positive	83 (43.0)	58 (48.3)	0	23 (54.8)	2 (14.3)			0.157	0.769
CK17	Negative	93 (48.4)	48 (40.0)	16 (100)	20 (47.6)	9 (64.3)				
	Positive	99 (51.6)	72 (60.0)	0	22 (52.4)	5 (35.7)			0.949	0.551
EGFR	Negative	67 (34.7)	31 (25.8)	17 (100)	12 (28.6)	8 (57.1)				
	Positive	126 (65.3)	89 (74.2)	0	30 (71.4)	6 (42.9)			0.437	0.852
CD117	Negative	124 (64.2)	66 (55.0)	13 (76.5)	31 (73.8)	14 (100)				
	Positive	69 (35.8)	54 (45.0)	4 (23.5)	11 (26.2)	0	0.002	0.04	0.06	0.003
p63	Negative	153 (79.3)	100 (83.3)	17 (100)	26 (61.9)	10 (71.4)				
	Positive	40 (20.7)	20 (16.7)	0	16 (38.1)	4 (28.6)	0.003	0.001	0.001	0.001
p53	Negative	92 (47.7)	63 (52.5)	7 (41.2)	16 (38.1)	6(42.9)				
	Positive	101 (52.3)	57 (47.5)	10 (58.8)	26 (61.9)	8 (57.1)	0.379	0.23	0.14	0.136
Vimentin	Negative	119 (61.7)	74 (61.7)	12 (70.6)	20 (47.6)	13 (92.9)				
	Positive	74 (38.3)	46 (38.3)	5 (29.4)	22 (52.4)	1 (7.1)	0.02	0.151	0.066	0.618
CK7	Negative	19 (9.9)	12 (10.0)	2 (11.8)	4 (9.8)	1 (7.1)				
	# score ≥3	173 (90.1)	108 (90.0)	15 (88.2)	37 (90.2)	13 (92.9)	0.98	0.971	0.931	0.813
CK8/18	Negative	13 (6.8)	8 (6.7)	4 (23.5)	1 (2.4)	0 (0.0)				
	# score ≥3	179 (93.2)	112 (93.3)	13 (76.5)	40 (97.6)	14 (100.0)	0.02	0.017	0.172	0.084
BCL2	Negative	121 (63)	70 (58.3)	16 (94.1)	21 (51.2)	14 (100.0)				
	# score >0	71 (37)	50 (41.7)	1 (5.9)	20 (48.8)	0 (0.0)	<0.001	0.008	0.185	0.911
p16	Negative	28 (14.6)	14 (11.7)	4 (23.5)	6 (14.6)	4 (28.6)				
	# score >0	164 (85.4)	106 (88.3)	13 (76.5)	35 (85.4)	10 (71.4)	0.25	0.395	0.806	0.371
	# score 3-6	83 (52.9)	58 (56.3)	7 (58.3)	11 (32.4)	7 (87.5)				
	# score 7-8	74 (47.1)	45 (43.7)	5 (41.7)	23 (67.6)	1 (12.5)	0.017	0.046	0.013	0.129
WT1	Negative	86 (44.8)	48 (40.0)	7 (41.2)	19 (46.3)	12 (85.7)				
	# score ≥3	106 (55.2)	72 (60.0)	10 (58.8)	22 (53.7)	2 (14.3)	0.013	0.776	0.48	0.041
WT1 nuclear stain	Negative	188 (97.9)	116 (96.7)	17 (100.0)	41 (100.0)	14 (100.0)				
	Positive	4 (2.1)	4 (3.3)	0 (0.0)	0 (0.0)	0 (0.0)	0.484	0.372	0.268	0.2
Cyclin D1	# score 0	47 (24.4)	28 (23.3)	9 (52.9)	9 (21.4)	1 (7.1)				
	# score >0	145 (75.1)	92 (76.7)	8 (47.1)	32 (76.2)	13 (92.9)	0.019	0.027	0.516	0.198

Abbreviations: TNB, triple negative basal-like; TNN, triple negative non-basal-like; TN, triple negative breast cancer; LumB, luminal B. #score=(intensity, 1-3)+(quantity,1-5); intensity, 1=mild, 2=intermediate, 3=strong; quantity, 0=no positive cell, 1=<1, 2=≥1-10, 3=≥10-33, 4=≥33-66, 5=≥66-100% positive cells

significant difference in high p16 expression between TNB and TNN. Diffuse strong expression was less in HER2+ subtype, and was significantly increased in lumB when compared with those with non-diffuse staining (Table 3, p16 score 3-6 vs. score 7-8).

Cyclin D1: All types with any positive tumor cell ranged from 47.1% in TNN to 92.9% in HER2+ subtype. Only 2.1% of all cases were strongly positive.

WT1: HER2+ subtype had the lowest cytoplasmic expression of WT1 (14.3% when using score 3 as the

cutoff), whereas TNB, TNN, and lumB subtypes had a similar proportion of positive WT1. Localization of WT1 in the nucleus was observed in only 4 patients belonging to the TNB group, and all of them died of their disease. Negative BCL2 was found in these 4 patients, and 3 of them had negative p16, luminal CKs, or cyclin D1. There was no association between cytoplasmic/cell membrane staining intensity or percentage of staining and nuclear staining of WT1.

Comparison of biomarker expressions between TN (TNB and TNN) and non-TN (lumB and HER2)

Ki67 index was not significantly different between the TN and non-TN groups. Increased CD117 expression was significantly found in TN, while p63 expression was more common in non-TN.

Survival analysis according to subtypes and biomarker expressions (Tables 4-5) (Figure 1).

There were 10 patients with distant metastasis at the time of diagnosis, so only 116 TNB, 14 TNN, 40 lumB, and 13 HER2 were analyzed for DFS. The mean follow-up time was 72.44±2.61 months. The median follow-up time was 62.93 months (range: 0.53-96.5 months). Local-regional recurrence occurred in 21 patients, and 53 patients had distant metastasis. Sixty-six deaths occurred during the follow-up period (Table 2).

The clinical parameters significantly associated with lower DFS and OS were tumor size larger than 5 cm, grade 3 tumor, and presence of axillary nodal involvement. Furthermore, patients with higher stage had significantly lower OS (Table 4).

DFS: When compared with TNB, TNN had significantly

Table 4. Clinicopathologic Features/breast Cancer Subtypes and Survivals

Clinicopathologic feature/Breast cancer subtype	DFS				OS			
	Case	Event	5-year survival	p	Case	Event	5-year survival	p
Age (years)								
≤50	91	30	0.687		96	32	0.699	
>50	92	34	0.66	0.767	97	34	0.671	0.808
Tumor size								
≤2 cm	63	19	0.702		64	19	0.741	
2-5 cm	108	37	0.696		114	36	0.711	
>5 cm	12	8	0.333	0.006	15	11	0.267	<0.001
Tumor grade								
1 & 2	47	22	0.515		49	22	0.562	
3	128	40	0.714	0.048	136	43	0.715	0.108
LVI								
No	115	32	0.757		120	32	0.762	
Yes	67	32	0.531	0.013	72	34	0.56	0.008
Axillary nodal involvement								
No	118	28	0.785		120	25	0.809	
Yes	65	36	0.473	<0.001	73	41	0.483	<0.001
Staging								
1, 2					150	36	0.787	
3, 4					43	30	0.33	<0.001
TNB vs TNN								
TNB	116	37	0.701	0.004	120	36	0.728	0.001
TNN	14	9	0.343		17	11	0.343	
TN vs LumB								
TN	130	46	0.661	0.296	137	47	0.679	0.389
LumB	40	12	0.749		42	12	0.737	
TN vs nonTN								
TN	130	46	0.661	0.514	137	47	0.679	0.722
nonTN	53	18	0.697		56	19	0.694	
TNB vs LumB								
TNB	116	37	0.749	0.53	120	37	0.735	0.606
LumB	40	12	0.701		42	12	0.737	

Abbreviations: TNB, triple negative basal-like; TNN, triple negative non-basal-like; TN, triple negative breast cancer; lumB, luminal B; LVI, lymphovascular invasion

Table 5. Biomarkers in TNB, TNN and TN Subtypes with Survivals

Subtype	Biomarker status		DFS				OS			
			Case	Event	5-year survival	p	Case	Event	5-year survival	p
TNB	CK5/6	-	36	10	0.771	0.522	37	9	0.829	0.354
		+	80	27	0.67		83	27	0.686	
	CK14	-	61	21	0.702	0.763	62	19	0.749	0.888
		+	55	16	0.699		58	17	0.706	
	CK5/14/17	-	22	5	0.816	0.262	22	5	0.909	0.295
		+	94	32	0.674		98	31	0.687	
	EGFR	# score 0,1	63	20	0.719	0.847	64	19	0.72	0.887
		2,3	53	17	0.677		56	17	0.739	
	EGFR/CKs	-	2	1	0.5	0.751	2	1	0.5	0.713
		+	114	36	0.704		118	35	0.733	
	CK7	# score ≤3	12	2	0.917	0.166	12	1	0.917	0.081
		>3	104	35	0.674		108	35	0.706	
	CK8/18	# score ≤3	8	2	0.75	0.565	8	2	0.75	0.601
		>3	108	35	0.696		112	34	0.726	
	P16	-	13	6	0.615	0.289	14	6	0.571	0.304
		+	103	31	0.711		106	30	0.749	
	WT1	-	44	13	0.717	0.62	45	11	0.778	0.251
		+	72	24	0.69		75	25	0.698	
Cyclin D1	-	27	11	0.619	0.247	28	11	0.679	0.227	
	+	89	26	0.726		92	25	0.743		
TNN	CK7	# score ≤3	2	2	0	<0.001	2	2	0	<0.001
		>3	12	7	0.4		15	9	0.289	
	CK8/18	# score ≤3	4	4	0	<0.001	4	4	0	0.001
		>3	10	5	0.48		13	7	0.449	
	p16	-	3	3	0	0.363	4	3	0.25	0.846
		+	11	6	0.455		13	8	0.385	
WT1	-	5	3	0.4	0.543	7	4	0.429	0.312	
	+	9	6	0.333		10	7	0.3		
Cyclin D1	-	8	6	0.25	0.102	9	6	0.333	0.712	
	+	6	3	0.5		8	5	0.375		
TN	CK7	# score ≤3	14	4	0.786	0.49	14	3	0.786	0.302
		>3	116	42	0.644		123	44	0.666	
	CK8/18	-	3	2	0.333	0.052	3	2	0.333	0.045
		+	127	44	0.699		134	45	0.687	
	CK8/18	# score ≤3	12	6	0.5	0.201	12	6	0.5	0.19
		>3	118	40	0.677		125	41	0.696	
	EGFR	-	44	21	0.516	0.014	47	23	0.506	0.005
		+	86	25	0.737		90	24	0.773	
	BCL2	# score 0-2	92	33	0.677	0.934	98	34	0.681	0.945
		>2	38	13	0.656		39	13	0.681	
	p16	-	16	9	0.492	0.085	18	9	0.494	0.204
		+	114	37	0.686		119	38	0.709	
WT1	-	49	16	0.682	0.497	52	15	0.728	0.196	
	+	81	30	0.649		85	32	0.649		
Cyclin D1	-	35	17	0.532	0.026	37	17	0.593	0.063	
	+	95	29	0.71		100	30	0.711		
All	CK8/18	-	3	2	0.333	0.03	3	2	0.333	0.032
		+	179	61	0.678		189	64	0.689	
	EGFR	-	63	27	0.584	0.08	67	29	0.58	0.047
		+	120	37	0.722		126	37	0.743	
	p16	-	25	13	0.517	0.06	28	14	0.497	0.069
		+	157	50	0.698		164	52	0.716	
CyclinD1	-	44	21	0.539	0.013	47	22	0.574	0.025	
	+	138	42	0.715		145	44	0.72		

Abbreviations: TNB, triple negative basal-like; TNN, triple negative non-basal-like; TN, triple negative breast cancer; lumB, luminal B; All, all cases. #score=(intensity,1-3)+(quantity,1-5); intensity, 1=mild, 2=intermediate, 3=strong; quantity, 0=no positive cell, 1=<1, 2=≥1-10, 3=>10-33, 4=>33-66, 5=>66-100% positive cells

lower DFS ($p=0.004$, Table 4; Figure 1). In TNN subtype, patients with a CK7 score ≤ 3 or CK8/18 score ≤ 3 who had disease recurrence before 5 years had significantly lower DFS ($p<0.001$) (Table 5). In the TN group, an absence of

EGFR or cyclin D1 was significantly associated with lower DFS ($p=0.014$ and $p=0.026$, respectively) (Table 5). OS: TNN had a significantly higher death rate than TNB ($p=0.001$, Table 4; Figure 1). No significant difference was observed when OS between lumB and TNB or between non-TN and TN were compared (Table 4). In TN, the patients with absence of EGFR or CK8/18 had significantly lower OS ($p=0.005$ and $p=0.045$, respectively, Table 5). Only one patient in the TN group had a cyclin D1 score ≥ 7 , and that patient died at 14.6 months. Four patients who had positive WT1 nuclear staining died, and three of those died before 15 months after diagnosis. Other biomarkers, including basal CKs, BCL2, p16 (in varying cut off levels), and WT1 (cytoplasmic staining), in each subtype and overall cases had no significant influence on survival (Table 5).

Effects of treatment on survival

Breast conserving surgery

Of 45 patients, 10 of 37 TNB, 1 of 2 TNN, 0 of 5 lumB, and 1 HER2+ had recurrence within 5 years, and 9 of 37 TNB, 1 of 2 TNN, 0 of 5 lumB, and 1 HER2+ died within 5 years ($p=0.191$ and $p=0.019$, respectively). However, the results of this analysis should be interpreted with caution due to the small number of subjects.

Total mastectomy

Of 145 patients with exclusion of stage 4 breast cancer, 37.8% of 82 TNB, 69.2% of 13 TNN, 37.8% of 37 lumB, and 46.2% of 13 HER2+ had recurrence within 5 years, and of 148 patients, 33.7% of 83 TNB, 66.3% of 15 TNN, 32.4% of 37 lumB, and 46.2% of 13 HER2+ died within 5 years. TNN had significantly lower DFS and OS when compared with TNB or lumB ($p=0.027$ and $p=0.021$, respectively). No significant difference in DFS or OS was observed between TNB and lumB.

Chemotherapy

TN patients and overall patients in our cohort who received chemotherapy had significantly increased DFS, but not significantly increased OS (all patients $p=0.028$ and $p=0.121$, respectively, TN patients $p<0.001$ and $p=0.114$, respectively). Among the overall cohort, anthracycline-based alone or in combination with taxane was used in 81% of patients who received neoadjuvant chemotherapy, in 77.8% who received postoperative chemotherapy, and in 82.3% who received neoadjuvant and postoperative chemotherapy (81.6% of TNB, and 78.6% of TNN) (Tables 2 and 4).

Radiotherapy

Patients who received radiotherapy had significantly lower DFS and OS ($p=0.03$ and $p=0.011$, respectively). In TN subtype, receiving radiotherapy did not result in significantly different DFS or OS. However, patients

with TN breast cancer who underwent lumpectomy and received subsequent radiotherapy had significantly increased DFS ($p=0.029$). In multivariate analysis, patients who received radiotherapy had borderline significantly increased DFS, but not OS (adjusted for age, types of surgery, and stages).

Multivariate analysis

Overall, multivariate analysis by Cox linear regression model showed that among clinicopathological parameters, tumor size and axillary nodal involvement were the independent prognostic factors for DFS (hazard ratio [HR]: 1.154, 95% confidence interval [CI]: 1.030-1.293, $p=0.013$, and HR: 2.497, 95% CI: 1.411-4.420, $p=0.002$, respectively), and for OS (HR: 1.162, 95% CI: 1.084-1.245, $p<0.001$, and HR: 2.628, 95% CI: 1.488-4.643, $p=0.001$, respectively). In TNN subtype, CK8/18 was the independent predictor for better DFS and OS (HR: 0.040, 95% CI: 0.002-0.758, $p=0.032$, and HR: 0.090, 95% CI: 0.011-0.717, $p=0.023$, respectively). In TN subtype, positive CK8/18 (score >0) and positive EGFR (score >0) were the independent predictors for DFS (HR: 0.045, 95% CI: 0.005-0.405, $p=0.006$, and HR: 0.491, 95% CI: 0.262-0.921, $p=0.027$, respectively). CK8/18 (score >0) and positive EGFR (score >0) were the independent predictors for OS (HR: 0.060, 95% CI: 0.007-0.512, $p=0.010$, and HR: 0.489, 95% CI: 0.263-0.909, $p=0.024$, respectively).

Discussion

This study, which had a larger sample size than our previous study, found 11.9% TN, of which 87.7% were TNB. This is quite different from the previous study that reported 25% TN, of which 56% were TNB [5]. This difference between studies may be explained by the larger size of our study cohort, differences in criteria for evaluating predictors or markers, and different clones of antibodies. The current positive criteria used were for the most part similar to those used in studies of IHC markers for basal-like breast cancer against a gene expression profile gold standard [10-12].

The prevalence of TNB over TNN in the current study was quite high (87.7%). With comparable thresholds of biomarker expression, this was similar to the finding of Taliano, et al. [13] (89.1%), lower (80.6% of 352 TN cases) than the finding of Levva, et al. [12], and lower (81.06% of 391 TN cases) than the finding of Prat, et al. [14]. We could find significant differences in DFS or OS between TNB and TNN, even with increased survival in the former. These findings were in agreement with those from a study by Choi, et al. [15], but contrary to the findings of other studies [16, 17]. The better prognosis of TNB could be related to its good response to chemotherapy [14] and its heterogeneity of disease [18]. This might suggest the need to differentiate the TNN from the TNB subgroup.

TNN also had the poorest prognosis among the four subtypes/subgroups (TNB, TNN, lumB, and HER2+), which was also reported by Choi, et al. [15]. Most lumB in our study had high Ki67 index, and approximately 10% of

those had *HER2* amplification. These findings may be explained by the archived pathology report diagnosis of TN breast cancer that was then revised to low positive ER or PR after our review. These types of lumB breast cancer have some phenotypes of TN breast cancer (high grade nuclei, high Ki67 index) that are considered to be subgroup with poor prognosis close to the TN cancer.

Regarding clinicopathological factors, the patients with TNN were significantly older than lumB breast cancer patients, and there was a trend toward TNN being older than TNB. Most patients in our cohort had high histologic grade, and all TNN were IDC, NOS whereas non-IDC, NOS including metaplastic and medullary-like carcinomas, were TNB subtype. There was no significant difference in tumor size, LVI, lymph node metastasis, or perinodal invasion among subtypes, except for TNN, which had a higher proportion of more than 3 metastatic axillary lymph nodes compared to lumB, and had the least perinodal extension. TNN also had higher staging (stages 3 and 4) and a higher death rate when compared with others. The higher staging of TNN could be one of the explanations for the observed worse prognosis.

Regarding the clinical prognostic factors, the commonly known adverse prognostic factors, tumor size larger than 5 cm and presence of axillary nodal involvement, were also the independent predictors for lower DFS and/or OS in this study. The presence of LVI or higher stage was associated with decreased OS.

Maximal tumor size was an adverse prognostic factor for both DFS and OS by univariate analysis in TNN if the tumor was more than 5 cm; however, in TNB, it only adversely affected OS.

Prevalence of biomarkers and their influence on survival in TNB, TNN, and TN

There is no single biomarker that is specific to any breast cancer subtype. Each can be more or less found in different subtypes, but their strength of expression varies. For example, basal CK expression is greater in TNB and lumB, but less in *HER2+*; EGFR is more commonly found in TNB, lumB, and *HER2+*; CD117 is more commonly found in TNB, TNN, and lumB; and p63 is more prevalent in non-TN than in TN. In our study, the combination of EGFR and CK5/6 can diagnose more than 98% of TNB. Thus, in routine practice, we suggest the using of both EGFR and CK5/6 for distinguishing between TNB and TNN.

All biomarker expressions studied in this cohort had no influence on either the DFS or OS of TNB, including basal CKs and EGFR at different cutoffs. This finding is similar to and different from some previous studies [15-17]. However, in some groups or combinations of subtypes, EGFR score >0, CK8/18 score >0, cyclin D1 score >0, and p16 score >0 were independent factors for DFS and/or OS.

EGFR expression is more commonly present in TNB than in other subtypes, and according to many studies is associated with adverse prognosis [19-21]. However, different biomarker cutoffs, even for hormone receptors, *HER2* status, and EGFR, have to be considered. In the

present study of biomarkers, EGFR expression was found in 74.2%, which is consistent with other studies that reported a range of 42-76% [19, 20, 22, 23], and close to the 71.6% reported from a study in the molecular activity and immunohistochemistry of EGFR [12]. That group also found the EGFR and EGFR/p53 immunophenotypes to be associated with favorable outcomes in TN patients receiving anthracyclines and/or taxanes. Using a comparable EGFR cutoff, our study also found the presence of an EGFR score >0 to be an independent factor for DFS and OS not only in the TN group, but also in the combined group of TN and lumB. These results may be explained by the adjuvant treatments with anthracycline-based and/or taxane in our cohort, which were as high as 81.6%, 78.6%, 84.8%, and 84.6% in the TNB, TNN, lumB, and *HER2+* groups, respectively.

Luminal CKs: The weak or absent expression of luminal CKs (CK 7, CK8, and CK18) were previously reported to be associated with recurrence/metastasis and OS [24-26]. Our study demonstrated that loss of CK7 or CK8/18 expression had an adverse effect on DFS and OS in TNN. In the TN group, loss of CK8/18 expression was also associated with lower OS, and a trend toward lower DFS. In multivariate analysis, all patients except for *HER2* subtype with a loss of CK8/18 expression had lower DFS and OS (all patients, including *HER2+*, had only decreased OS). These findings also support those from the previous studies. The explanation for this could be related to the dedifferentiation of tumor cells leading to proliferation and progression of the tumor [24, 26].

Among all luminal CKs (CK7, CK8/18, and CK19), loss of CK19 expression was found in 3.1% of the patients, while loss of CK8/18 was found in 1.5% of patients. As such, when using CK19 as the primer in polymerase chain reaction for detection of metastatic carcinoma in sentinel lymph node, there could be some false negativity, especially in high-grade carcinoma.

BCL2: The protein product of the *BCL2* (B-cell lymphoma-2) gene is commonly known as its anti-apoptotic activity. The *BCL2* family includes anti-apoptotic regulators, pre-apoptotic regulators, and antiapoptotic proteins. Interactions among this family of proteins could result in cell apoptosis [27, 28]. In breast cancer, the expression ranged upward to 54.4% [29], with the lowest expressions of 11.4-18.3% observed in *HER2+* subtype [29, 30] and commonly in *HR+/HER2-* [29]. In our study of the TN group, we found low or no expression in the TNN and *HER2+* subtypes, which is similar to a previous study that found no expression in *HER2+* subtype, and less presence in TNN than in TNB subtype [31]. The expression of *BCL2* in TN (predominantly in TNB) was 37.3% or 20.4% according to different cutoffs. The frequency of the expression was comparable to that reported from other studies [30, 32]. Regarding its effect on survival, there is some controversy regarding the role of *BCL2* as a prognostic marker. It was shown to be a favorable prognosticator in overall breast cancers, and an independent prognostic marker in *HR+/HER2-* subtype [29, 30]. Multivariate analysis in another study revealed independent association between *BCL2* and unfavorable

outcome in the non-basal subgroup [32], and in TN patients treated with anthracycline-based adjuvant chemotherapy [21]. In the present study, we found no prognostic influence of this protein on TNB or lumB.

These findings are in agreement with those reported from two previous studies [29, 30]. In the present study, we found no significant influence of BCL2 on survival among the overall cohort, or in any of the subtypes.

p16: This marker is a tumor suppressor protein that acts as a cyclin-dependent kinase (CDK) inhibitor that inactivates CDK4/6, which inhibits phosphorylation of the retinoblastoma protein and leads to arrest of the cell cycle. Our study demonstrated high expression of p16 in lumB without any prognostic significance, whereas loss of expression was an independent prognostic indicator for lower DFS and OS among the whole cohort. This is in agreement with a study in breast cancer cell line that postulated that loss of p16 expression could reduce the response of ER-negative breast cancer cells to chemotherapy by conferring cancer stem cell-like properties [33]. Another study reported association between diffusely positive p16 and increased survival in no-special type TN patients treated with neoadjuvant chemotherapy [34]. We did not find this association. Our group is currently investigating the relationship between p16 expression and in retinoblastoma proteins in this cohort.

Cyclin D1: This marker is a protooncogene expression that is present in and shorting the G1 phase of the cell cycle via the cyclin D1 and CDK 4/6 complex pathway resulting in uncontrolled proliferation. It also interacts with a variety of other transcription factors, including ER, androgen receptor, and histone deacetylases and acetylases, which suggests that cyclin D1 plays an important role in the regulation of transcription [35, 36]. Cyclin D1 was shown to express in about half of all invasive carcinomas [37], and it was found to positively correlate with ER and HER2 expression [38]. In our cohort, it was expressed in 47.1% of TNN, and in 92.9% of HER2+. The results of cyclin D1 expression were not different from the previous reports. The prognostic/predictive role of cyclin D1 expression has not yet been fully elucidated. Higher expression of cyclin D1 was shown to improve tumor-free survival [38]. Alternatively, higher expression of cyclin D1 was reported to be associated with increased risk of breast cancer death in ER positive cases [39]. Regarding ER-negative breast cancer, there is still some controversy on overexpression of cyclin D1 with poor or good DFS and OS [40, 41]. In univariate analysis, cyclin D1 expression was significantly associated with better DFS in TN, and with better DFS and OS among all cases in our entire cohort without HER2+; however, those significant associations did not survive multivariate analysis. With other cutoffs, there was no significant correlation. Comparison with others or conclusion of the outcome results were quite difficult as there was some difference in the cutoffs and the treatments used.

WT1: This marker is a product of WT1 (Wilms' tumor), which was originally known as tumor suppressor gene [42]. It has a role in proliferation, angiogenesis, and

cancer induction epithelial-to-mesenchymal transition [43]. Whether it acts as an oncogenic or tumor suppressing factor in breast cancer is still unclear. Expression of this gene was found in 10-30% of breast cancer, and it showed increased expression in ER-positive tumors [43]. WT1 cytoplasmic protein expression was detected in 48.5%, and was found to be more prevalent in ER-positive tumors [44]. Nuclear staining was much less expressed in up to 7% [45]. WT1 expression was reported to be associated with both improved [46] and worse outcomes [47]. In a recent study by Artibani [43], it was concluded that WT1 played a role in regulating the epithelial-mesenchymal balance of breast cancer cells, and that WT1-expressing tumors were mainly associated with a mesenchymal phenotype. WT1 expression was correlated with CYP3A4 levels, and associated with poorer response to taxane treatment [43]. In our study, which was limited to TN, cytoplasmic expression of WT1 was found in 55.2% of all patients of the cohort, predominantly in TN and lumB. Less expression of both WT1 and vimentin was found in HER2+. This may be explained by WT1's role in epithelial-mesenchymal transition. However, we could not find any correlation between cytoplasmic expression and the outcome in any subgroup. For the nuclear staining, it was found in only 4 TNB and all died of their disease. Although the number of positive cases was small, all had poor OS. They had negative p16 and negative luminal CK which were associated with poor outcomes according to our study.

Effects of treatment on survival

Due to the small number of patients who underwent breast-conserving treatment (37 TNB and 2 TNN), comparison between these two subtypes could not be confidently performed. Our study confirmed the poorer prognosis of TNN over TNB or lumB in patients who underwent total mastectomy. There was no significant difference in DFS or OS between TNB and lumB. According to the European Society for Medical Oncology (ESMO), cytotoxic chemotherapy is the standard treatment for TN, and the choice of regimen should be determined after consideration of several disease-related factors (histology, biomarkers, staging, previous therapies, and response) and patient-related factors (patient preference, biological age, menopausal status, comorbidities, and socioeconomic and psychological factors) [48, 49]. Cytotoxic chemotherapy, especially taxane and anthracycline-based combination chemotherapy, has been the standard treatment for TN [48, 50]. In our study, 81.6% of TNB and 78.6% of TNN received these chemotherapy regimens. TN patients and overall patients in our cohort who received chemotherapy had significantly increased DFS, but not OS. The patients in our cohort received standard treatment and the result of the outcome could be comparable to others.

Patients who received radiotherapy had significantly lower DFS and OS. This might be due to radiotherapy administration in more advanced loco-regional disease. TN breast cancer patients who underwent breast-conserving surgery and who received radiotherapy had significantly increased DFS. Multivariate analysis revealed that patients

who received radiotherapy had borderline significantly increased DFS, but not OS. This finding supports the role of radiotherapy as local control for TN subtype breast cancer.

In conclusion, among TN breast cancers, the incidence of TNN in Thai patients was 12.3%, which is much lower than that reported from Western countries, and the prognosis was significantly poorer when compared with TNB. Tumor size and axillary nodal involvement were the independent predictors for DFS and OS. Among the biomarkers studied, absence of EGFR expression was an independent factor for lower DFS and OS in TN, and in combined TN and lumB subtype. Absence of either CK8/18 or p16 expression was an independent factor for decreased DFS of the whole cohort, and for decreased OS in the combined TN and lumB group.

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Conflict of interest declaration

All authors declare no personal or professional conflicts of interest, and no financial support from the companies that produce and/or distribute the drugs, devices, or materials described in this report.

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