

Expression of androgen receptor and its regulatory molecule Lin28 in non-luminal subtype breast cancer

DOONYAPAT SA-NGUANRAKSA¹, WANEE PONGTHONG^{1,2}, NORASATE SAMARNTHAI³,
KWANLADA MITPAKDI^{1,2}, TUENJAI CHUANGSUWANICH³, THAWORNCHAI LIMJINDAPORN²,
ANCHALEE KULPROM¹ and PORNCHAI O-CHAROENRAT¹

¹Division of Head Neck and Breast Surgery, Department of Surgery; Departments of ²Anatomy and ³Pathology,
Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand

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Abstract. Androgen receptor (AR) was associated with favourable outcome in luminal breast cancer. However, the role of AR in non-luminal breast cancer remains inconclusive. The aim of the present study was to evaluate the clinical significance of the AR and its regulatory pathway in non-luminal subtypes of breast cancer. In total, 284 breast cancer patients were recruited from January 2007 to January 2016. Tissue microarrays were constructed from archival paraffin blocks and assessed for AR and its regulatory molecule, Lin28, by immunohistochemistry. The association between AR and Lin28 expression and clinicopathological parameters was analyzed. Results showed that AR and Lin28 were co-expressed. No association between these proteins and clinicopathological parameters, and survival outcome was found. However, a higher proportion of the patients with AR and Lin28 expression were observed in HER2 subtype. In conclusion, Lin28 may be a novel marker for prognosis and targeted for treatment in HER2 subtype breast cancer.

Introduction

Breast cancer is the most common malignancy in women worldwide, including Thailand, accounting for 25% of all cancers in women (1,2). Hormone receptor (HR)-negative breast cancer or non-luminal breast cancer patients have a poorer outcome than other subtypes and lack of hormonal therapy for long-term control of the disease. Non-luminal breast cancer comprises triple negative breast cancer (TNBC) and HER2 subtypes. Due to a lack of target for

therapy, systemic chemotherapy is the main treatment for TNBC. In addition, approximately 20-50% of HER2-positive patients showed resistance to Trastuzumab one year after treatment (3). Therefore, identification of novel prognostic markers and alternative treatments is imperative for both subtypes.

Androgen receptor (AR), a class I steroid receptor is commonly expressed up to 70% in primary breast cancer and approximately 50-75% in metastatic breast cancer (4,5). In luminal subtype breast cancer, AR co-expression was associated with better outcomes (6,7). AR expression was a significant prognostic factor for disease-free survival (DFS), overall survival (OS) and decreased risk of metastasis of non-luminal subtype breast cancer in some studies (8-10). By contrast, androgen can induce proliferation of AR-positive/estrogen receptor (ER)-negative cells as commonly found in molecular apocrine subtype which had AR expression of approximately 50% (11-13). Previous studies reported that RNA binding protein, Lin28A (referred to as Lin28 in this study), which regulates let-7 miRNA, stimulates HER2 expression and alters AR promoter activity (14-16). The upregulation of Lin28 in adults leads to carcinogenesis and progressive cell proliferation in several malignancies, including breast cancer (17-20). However, the role of these proteins in non-luminal breast cancer remains controversial. The aim of the present study was to evaluate the clinical significance of AR and Lin28 in non-luminal subtype breast cancer. It was found that AR and Lin28 are co-expressed. Thus, Lin28 may be a novel marker for prognosis and targeted for treatment in HER2 subtype breast cancer.

Patients and methods

Patients and data collection. In total, 284 patients were retrospectively recruited at the Division of Head Neck and Breast Surgery, Department of Surgery, Faculty of Medicine, Siriraj Hospital, Mahidol University (Bangkok, Thailand) from January 2007 to January 2016. The patients with pathological stage I-III, invasive ductal breast carcinomas, age at diagnosis equal to or more than 20 years, and HR negative were included. The sample size was determined by the formula for estimation of infinite population proportion using parameters

Correspondence to: Dr Pornchai O-Charoenrat, Division of Head Neck and Breast Surgery, Department of Surgery, Faculty of Medicine, Siriraj Hospital, Mahidol University, 2 Wanglang Road, Bangkok 10700, Thailand
E-mail: pornchai.och@mahidol.ac.th

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from a previous study (15). The proportions of Ki-67 status were 0.375 and 0.197 in groups 1 and 2. The ratios of proportion in both groups were 2.590 (according to AR expression), $\alpha=0.05$, 2-sided test, and power 80%. This resulted in a sample size of 239. To achieve statistically significant difference and the expected 10% dropout of the patients, the total sample size was approximately 250 cases. The current study was approved by the ethics committee of the Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand (COA no. Si733/2016). This study was performed in a retrospective manner, therefore, no informed consent was obtained from the patients.

The data collected from medical records comprised age at diagnosis, pathological reports, surgical procedure, adjuvant treatment (chemotherapy, hormonal therapy, targeted therapy, and radiotherapy), and follow-up data. Repository formalin fixed-paraffin embedded (FFPE) breast cancer and non-neoplasm control tissues (prostate, tonsil, and testis) in excess of standard pathological examination were obtained from the Department of Pathology, Faculty of Medicine, Siriraj Hospital, Mahidol University. The case record forms did not indicate any identification that linked to individual patients.

Tissue microarray (TMA). All H&E-stained slides and corresponding paraffin blocks of each case including non-cancerous breast tissues were collected and reviewed. The selected areas mapped on donor paraffin blocks were punched by manual microarrayer with diameter 2 mm for 3 cores and placed into the applied recipient mold. Each mold was melted at 60°C for 6 min and re-embedded. Finally, each slide contained triplicate of 17 cases, negative, and positive tissue controls. The TMA blocks were sliced into 4 μ m thickness. The section ribbon was placed on the slide glass and air dried for 30 min.

Immunohistochemistry. Expression of AR and Lin28 was assessed by immunohistochemistry (IHC) using mouse monoclonal anti-human AR (AR441, dilution 1:300, Dako) and mouse monoclonal anti-human Lin28A (55CT58.12.1, dilution 1:75, Sigma-Aldrich). AR staining was performed by semi-autostainer (Agilent Technologies, Dako; Autostainer Link 48). Deparaffinization, rehydration, and antigen retrieval were performed by target retrieval solution high pH (pH 9.0) at 95°C with PT Link (Dako PT link). Lin28 staining was performed by manual procedure. PT Link (Dako PT link) with target retrieval solution high pH (pH 9.0) was used. The sections were incubated overnight at 4°C with primary antibody. Subsequently, the sections were warmed up at room temperature (25°C) and rinsed twice with PBS. Peroxidase-blocking solution (Dako Peroxidase Blocking Code SM801) was used for endogenous blocking for approximately 5 min and then rinsed twice with PBS for 10 min. The sections were incubated with HRP-conjugated secondary antibody (Envision FLEX-HRP Code SM802) for 20 min. The visualization step was performed with Envision FLEX DAB and Chromogen (Envision FLEX DAB and Chromogen Code DM827) for 12 min and then rinsed with tap water for 5 min. The sections were counterstained with hematoxylin. Finally, the sections were dehydrated with

alcohol series (95 and 100% alcohol and acetone, respectively) and cleared with xylene.

The protein expression level was calculated by a mean score of 3 cores. The AR-positive status was determined by an established cut-off value of >20% of nucleus staining. Lin28 status was evaluated by cytoplasmic staining and scored according to the Modified Allred Scoring system including staining intensity and percentage of positive cells. The sum of staining and percentage was classified as: 0-2, negative and 3-8, positive. Scoring was performed by two experienced breast pathologists who did not know the clinical data of patients.

Dual in situ hybridization (DISH). For HER2 equivocal cases (IHC score 2+), HER2 amplification status was assessed by dual color *in situ* hybridization (DISH). The process was performed by using a cocktail-specific probe for HER2 and chromosome 17 (Chr 17) on a single slide. The HER2 copies were detected using the HER2 DNP-labeled probe and visualized via ultraView SISH detection kit [Ventana ultraView SISH dinitrophenyl (DNP), Ventana Medical System, USA]. Centromeres of chromosome 17 were assigned by Chr17 DIG-labeled probe and visualized by ultraView Red ISH detection kit [Ventana ultraViewRed ISH digoxigenin (DIG), Ventana Medical System]. DISH staining was performed by auto-staining system (BenchMark XT automated slide stainer, Ventana). The black signal (HER2) to red signal (Chr 17) ratio was manually counted by light microscope at a magnification, x20 for 20 cells and calculated. The ratios of equal or more than 2.0 were considered as HER2 amplification.

Statistical analysis. Associations between protein expression and clinicopathological parameters were analyzed using a Chi-square test. Binary logistic regression was performed for multivariate analysis using backward conditional method. Survival analysis was performed by Log-rank test and survival curves were estimated by Kaplan-Meier method. The DFS time was calculated from the date of surgery to the date of cancer recurrence, metastasis or death. The OS time was calculated from the date of surgery to the date of death. The Cox proportional hazards model was applied for prediction of survival rate. Multivariate analysis was performed by Cox regression to evaluate the effect of independent prognostic factors on DFS and OS. The SPSS software version 21 was used for statistical analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Patient characteristics. A total of 284 patients were eligible and recruited in this study. Patient characteristics are presented in Table I. HER2 equivocal cases by IHC were further assessed for HER2 amplification by DISH (Fig. 1). The mean age at diagnosis was 55.39 years (± 11.36 years). There were 131 HER2 subtype breast cancer patients and 24 patients receiving HER2-targeted therapy. TNBC subtype was 153 cases. Two hundred and two patients (71.1%) were post-menopause. The mean tumor size was 20.9 mm (± 10.5 mm). A tumor size >20 mm was found in 190 cases

Table I. Demographics data of non-luminal subtype patients.

Parameters	Number, n=284 (%)
Age at diagnosis	55.39 (\pm 11.36)
Age at diagnosis [n (%)]	
\leq 50 years	87 (30.6)
>50 years	197 (69.4)
Tumor size [n (%)]	
\leq 20 mm	94 (33.1)
>20-50 mm	172 (60.6)
>50 mm	18 (6.3)
Histological grading [n (%)]	
II	93 (32.7)
III	191 (67.3)
Lymphovascular invasion [n (%)]	
Absence	201 (70.8)
Presence	83 (29.2)
Axillary nodal metastasis [n (%)]	
No	166 (58.5)
Yes	118 (41.5)
N stage [n (%)]	
N0	166 (58.5)
N1	59 (20.8)
N2	28 (9.9)
N3	31 (10.9)
Staging [n (%)]	
I	73 (25.7)
II	143 (50.4)
III	168 (23.9)
HER-2 status [n (%)]	
Negative	153 (53.9)
Positive	131 (46.1)
HER-2 targeted therapy [n (%)] ^a	
No	107 (81.7)
Yes	24 (18.3)

^aIn HER2-positive patients. All patients received chemotherapy according to clinical practice guidelines.

(66.9%). Approximately half of the patients were in stage II at diagnosis (143 cases, 50.4%). There was no grade I tumor while the majority of the patients had grade III tumor (67.3%). All the patients received chemotherapy according to clinical practice guidelines and completed the course of treatment.

AR and Lin28 expression. AR was expressed in 66 of 284 non-luminal tumors (23.2%). AR expression was detected in 45 (68.2%) and 21 cases (31.8%) in HER2 and TNBC subtype, respectively. Lin28 protein was detected in 201 out of 284 patients (70.8%). From these, 164, 34, and 3 patients had weak, moderate, and strong AR staining, respectively. Ninety-two TNBC and 109 HER2 patients had Lin28 expression (Figs. 2-3).

Association between protein expression and clinicopathological parameters. Among 284 patients, AR and Lin28 status was significantly associated with HER2-positive status. In addition, both proteins were co-expressed together in non-luminal breast cancer (Tables II-III). Multivariate analysis revealed that AR-positive status was associated with the absence of axillary lymph node metastasis (OR=0.428, 95% CI 0.224-0.817, P=0.010), HER2-positive (OR=2.948, 95% CI 1.555-5.587, P=0.001), and Lin28-positive status (OR=15.756, 95% CI 3.707-66.979, P<0.001). Lin28 expression was associated with HER2-positive (OR=2.562, 95% CI 1.426-4.603, P=0.002), and AR positive status (OR=15.437, 95% CI 3.649-65.312, P<0.001).

Survival analysis. The median follow-up time was 43 months (1-139 months). There were 51 events that occurred during follow up including 3 loco-regional recurrences, 12 metastases, and 36 deaths. Two hundred and thirty-three patients were alive without disease. Univariate analysis via log-rank test revealed that tumor size, pathological staging, axillary lymph node metastasis, and lymphovascular invasion (LVI) were associated with lower DFS (P=0.042, P<0.001, P<0.001 and P<0.001, respectively). Pathological staging, axillary lymph node metastasis, and LVI were associated with lower OS (P<0.001, P<0.001 and P<0.001, respectively). Multivariate analysis revealed that pathological stage and LVI were the strong independent factors for DFS (HR=2.769, 95% CI 1.383-5.544, P=0.004 and HR=2.748, 95% CI 1.391-5.428, P=0.004, respectively) and OS (HR=3.160, 95% CI 1.347-7.415, P=0.008 and HR=3.615, 95% CI 1.533-8.525, P=0.003, respectively). The survival curves among HER2 and TNBC subtypes by AR and Lin28 status are shown in Fig. 4. There was no significant difference in survival among different AR and Lin28 status.

Discussion

The present study demonstrated the associations between the expression of AR and Lin28 in non-luminal breast cancer. In HER2-overexpressed breast cancer, we also showed the association between the expression of Lin28 and HER2.

A higher proportion of AR expression was observed in HER2 subtype in the present study. Similar studies by Micello *et al* (21) and Park *et al* (22), showed that AR expression was often detected in ER-negative/HER2-positive breast cancer. The implications of HER2 and AR have been suggested in molecular basis. HER2 is a transcriptional target of AR and able to activate ERK activity (11,12). *In vitro* studies suggested that androgen can induce proliferation in AR-positive/ER-negative cells such as those commonly found in the molecular apocrine subtype which exhibited AR co-expression of approximately 50% (3,13). He *et al* reported that treatment with Enzalutamide, an AR antagonist, reduced the ability of tumor growth via decreased cell proliferation and increased cell death in HER2-positive breast cancer, both *in vitro* and *in vivo* (3). AR-positive/ER-negative in HER2 overexpression or amplification in breast cancer has been reported to be associated with unfavourable outcome when compared to those with AR-negative (5,22-24). However, in the present study, we did not find any significant association

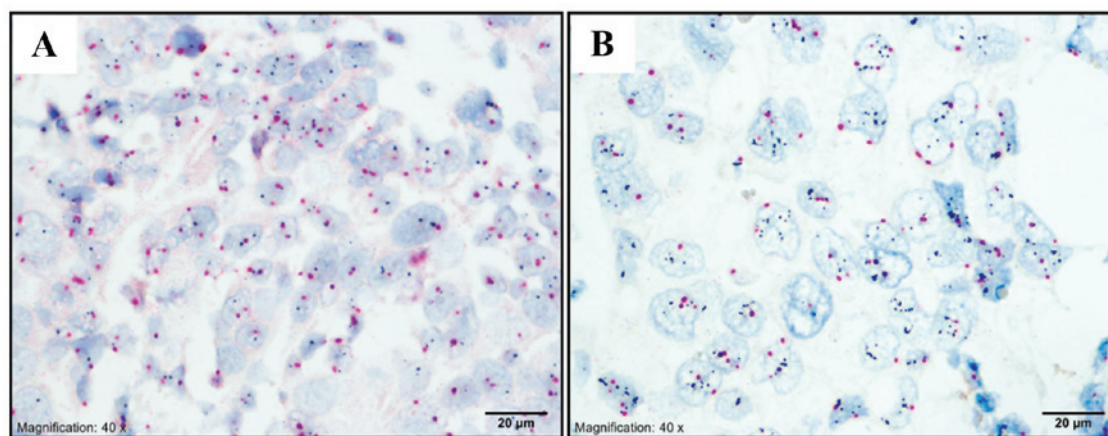


Figure 1. HER2 DISH was used to assess HER2 amplification in the patients with equivocal HER2 immunohistochemistry. (A) Breast cancer cells with a low level of HER2 signal. (B) Breast cancer cells with HER2 amplification.

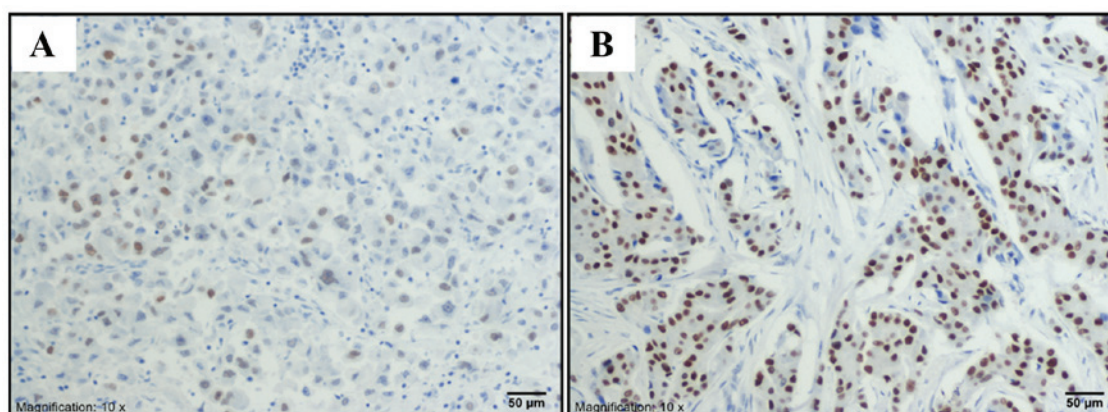


Figure 2. Immunostaining of AR. (A) AR-negative status, percentage of cells staining range, 0-20%. (B) AR-positive status, percentage of cells staining >20%. Magnification, x10.

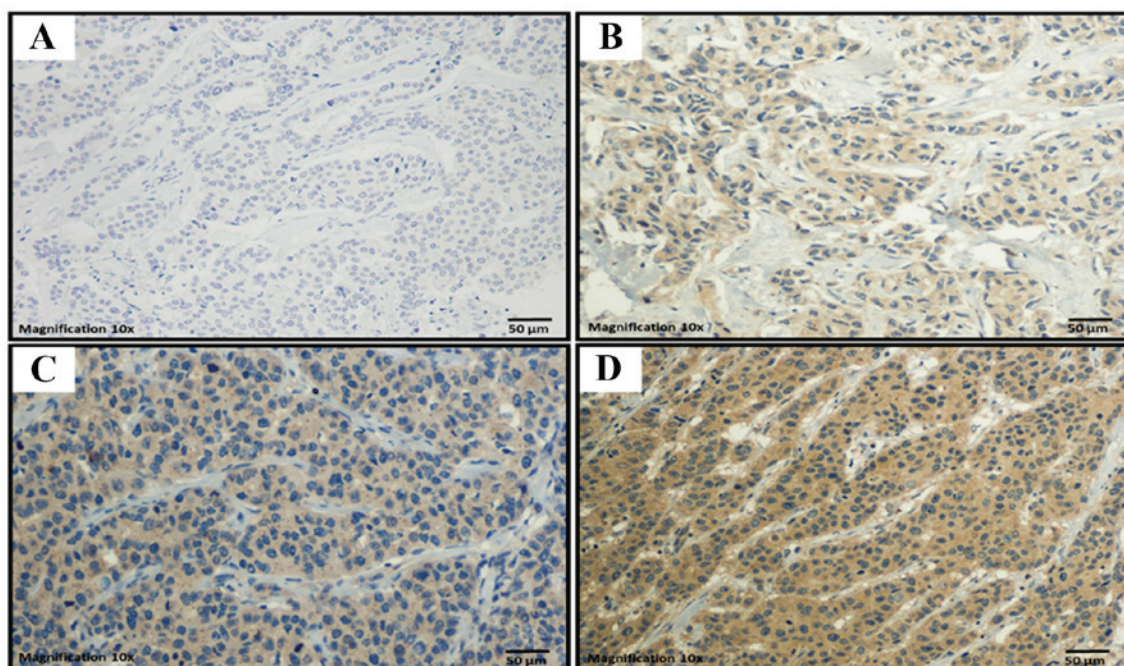


Figure 3. The cytoplasmic staining levels of Lin28. (A) Negative staining, (B) weak staining: 1+, (C) moderate staining: 2+, (D) strong intensity: 3+. Magnification, x10.

Table II. Associations between AR expression and clinicopathological parameters in non-luminal subtype.

Clinicopathological parameters	AR (n=284)				Multivariate analysis ^a		
	Negative, n (%)	Positive, n (%)	OR (95% CI)	P-value	Exp (B)	95% CI	P-value
Age (years)							
≤50	69 (31.7)	18 (27.3)	1 (ref.)	0.499	1.106	0.562-2.176	0.770
>50	149 (68.3)	48 (72.7)	1.235 (0.669-2.278)				
Tumor size (mm)							
≤20	66 (30.3)	28 (42.4)	1 (ref.)	0.068	0.705	0.369-1.348	0.291
>20	152 (69.7)	38 (57.6)	0.589 (0.334-1.039)				
Histological grading							
II	70 (32.1)	23 (34.8)	1 (ref.)	0.678	1.030	0.532-1.996	0.930
III	148 (67.9)	43 (65.2)	0.884 (0.495-1.580)				
Pathological staging							
I, II	166 (76.1)	53 (80.3)	1 (ref.)	0.482	1.325	0.495-3.545	0.575
III	52 (23.9)	13 (19.7)	0.783 (0.396-1.549)				
Axillary node metastasis							
No	121 (55.5)	45 (68.2)	1 (ref.)	0.069	0.428	0.224-0.817	0.010 ^b
Yes	97 (44.5)	21 (31.8)	0.582 (0.325-1.043)				
Perinodal invasion (pN+ patients)							
Absent	49 (50.5)	12 (57.1)	1 (ref.)	0.582	0.643	0.233-1.779	0.396
Present	48 (49.5)	9 (42.9)	0.766 (0.300-1.983)				
Perineural invasion							
Absent	207 (95.0)	63 (95.5)	1 (ref.)	0.869	0.661	0.164-2.667	0.561
Present	11 (5.0)	3 (4.5)	0.896 (0.242-3.312)				
LVI							
Absent	150 (68.8)	51 (77.3)	1 (ref.)	0.187	0.919	0.418-2.024	0.835
Present	68 (31.2)	15 (22.7)	0.649 (0.341-1.234)				
HER2 status							
Negative	132 (60.6)	21 (31.8)	1 (ref.)	<0.001 ^b	2.948	1.555-5.587	0.001 ^b
Positive	86 (39.4)	45 (68.2)	3.289 (1.833-5.903)				
Lin28 status							
Negative	81 (37.2)	2 (3.0)	1 (ref.)	<0.001 ^b	15.756	3.707-66.979	<0.001 ^b
Positive	137 (62.8)	64 (97.0)	18.92 (4.510-79.373)				

^aMultivariate analysis shows the variables in the equation at the last step before removal from the model. ^bStatistically significant. AR, Androgen receptor; LVI, lymphovascular invasion; OR, odds ratio; 95% CI, 95% confident interval.

between AR expression and unfavourable clinicopathological parameters or worse survival outcomes.

One of the main AR transcriptional regulatory cascades involves let-7 and Lin28. Lin28 is an RNA-binding protein (RBP) that directly regulates let-7 miRNA. Aberration thereof could lead to carcinogenesis and progressive cell proliferation in breast cancer (25). In the HER2 subtype, the association between AR expression and Lin28-positive status was detected in the present study. Lin28 regulates the expression of AR via c-myc, a proto-oncogene involved in cell proliferation (16). The study by Feng *et al* also demon-

strated the relationship between Lin28 expression and ER negative/HER2-positive in breast cancer cell (14). The Lin28 responsive element (LRE) is 200 nucleotides in length and is located in nearly 5' end of the coding region of HER2 mRNA. The authors suggested that HER2 mRNA contains a cis-acting element that is specifically recognized by Lin28 within the coding region and activates translation in breast cancer. An 'A' bulge flanked by two GC base pairs in the secondary structure of HER2 mRNA served as the binding site for Lin28 (26). Shen *et al*, reported that Lin28 and AR were co-expressed in ER-negative/HER2-positive breast

Table III. Associations between Lin28 expression and clinicopathological parameters in non-luminal subtype.

Clinicopathological parameters	Lin28 (n=284)				Multivariate analysis ^a		
	Negative, n (%)	Positive, n (%)	OR (95% CI)	P-value	Exp (B)	95% CI	P-value
Age (years)							
≤50	28 (33.7)	59 (29.4)	1 (ref.)	0.467	0.927	0.503-1.709	0.808
>50	55 (66.3)	142 (70.6)	1.225 (0.709-2.117)				
Tumor size (mm)							
≤20	23 (27.7)	71 (35.3)	1 (ref.)	0.216	0.743	0.403-1.368	0.340
>20	60 (72.3)	130 (64.7)	0.702 (0.401-1.230)				
Histological grading							
II	24 (28.9)	69 (34.3)	1 (ref.)	0.377	0.893	0.484-1.650	0.718
III	59 (71.1)	132 (65.7)	0.778 (0.446-1.358)				
Pathological staging							
I, II	66 (79.5)	153 (76.1)	1 (ref.)	0.536	1.298	0.635-2.655	0.474
III	17 (20.5)	48 (23.9)	1.218 (0.653-2.273)				
Axillary node metastasis							
No	49 (59.0)	117 (58.2)	1 (ref.)	0.898	1.065	0.483-2.344	0.877
Yes	34 (41.0)	84 (41.8)	1.035 (0.615-1.740)				
Perinodal invasion (pN+ patients)							
Absent	19 (55.9)	42 (50.0)	1 (ref.)	0.563	1.196	0.553-2.583	0.650
Present	15 (44.1)	42 (50.0)	1.267 (0.569-2.821)				
Perineural invasion							
Absent	81 (97.6)	189 (94.0)	1 (ref.)	0.223	2.324	0.479-11.290	0.296
Present	2 (2.4)	12 (6.0)	2.571 (0.563-11.750)				
LVI							
Absent	56 (67.5)	145 (72.1)	1 (ref.)	0.432	0.823	0.450-1.506	0.527
Present	27 (32.5)	56 (27.9)	0.801 (0.461-1.393)				
HER2 status							
Negative	61 (73.5)	92 (45.8)	1 (ref.)	<0.001 ^b	2.562	1.426-4.603	0.002 ^b
Positive	22 (26.5)	109 (54.2)	3.285 (1.875-5.756)				
AR status							
Negative	81 (97.6)	137 (68.2)	1 (ref.)	<0.001 ^b	15.437	3.649-65.312	<0.001 ^b
Positive	2 (2.4)	64 (31.8)	18.920 (4.510-79.373)				

^aMultivariate analysis shows the variables in the equation at the last step before removal from the model. ^bStatistically significant. LVI, lymphovascular invasion; OR, odd ratio; 95% CI =95% confident interval.

cancer tissues and cell lines, suggesting a worse survival outcome (15,16).

In the present study, the positive association between AR and Lin28 was noted. However, only one-third of positive Lin28 breast cancer patients have positive AR expression. In ER-negative/HER2-positive breast cancer, several signaling cascades including the upregulation of Wnt and c-myc were involved in the interaction between AR and HER2 (12,27). Lin28 can activate the proliferation and growth of tumor cells via Lin28/let7 pathway (20). Patients with Lin28 expression

tended to have a lower survival rate compared to patients without Lin28 expression in HER2 subtype. This result was in accordance with previous studies regarding the potential role of Lin28 in decreased tumor suppressor miRNA and increased oncoproteins in breast cancer (15,16,18,28-30).

In conclusion, this current results have demonstrated the role of Lin28 in HER2-overexpressed breast cancer and showed the potential prognostic factor. Thus, Lin28 may be a novel marker for prognosis and future-targeted therapy for HER2 subtype breast cancer.

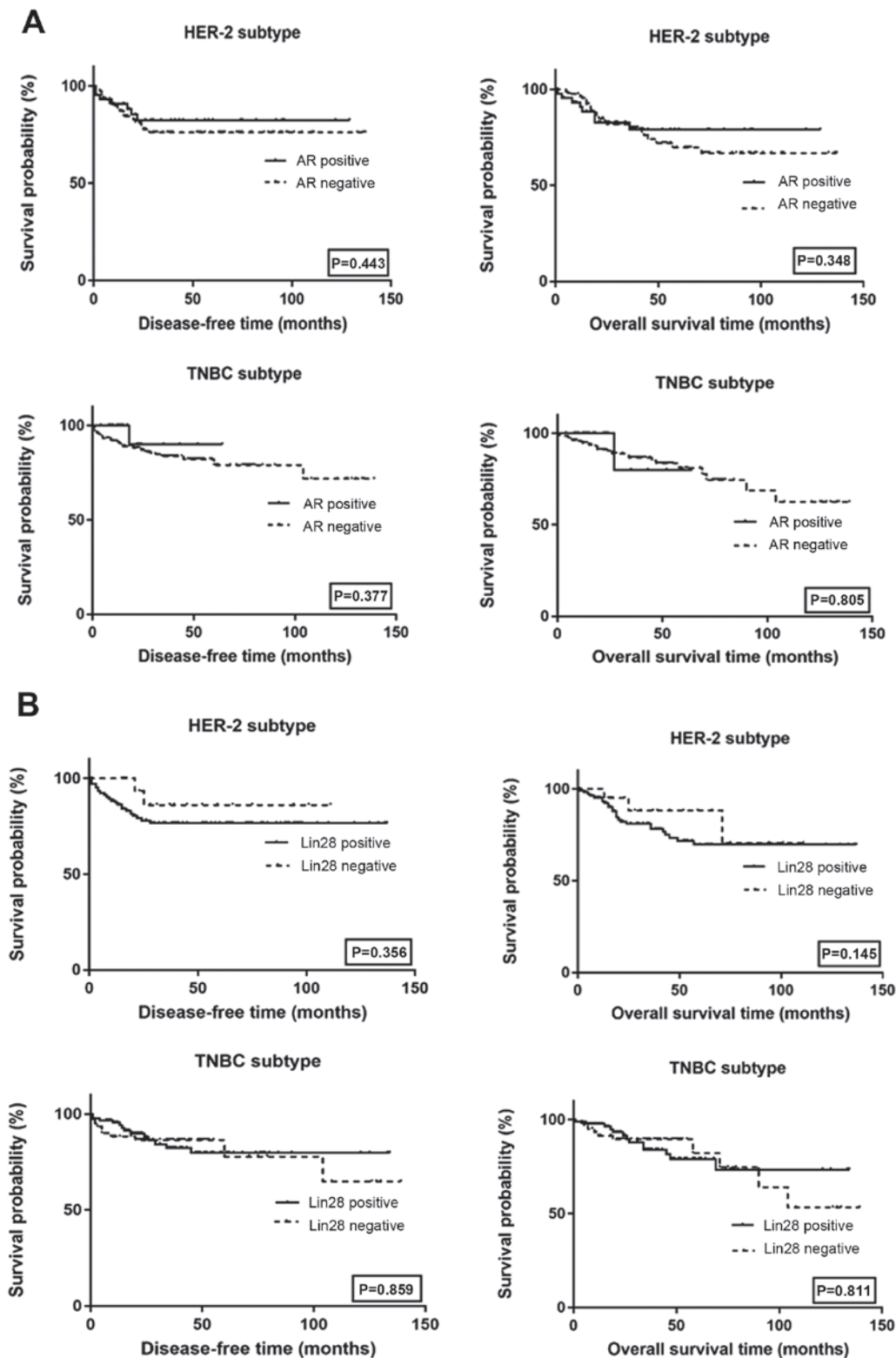


Figure 4. Survival curves of HER2 and TNBC subtypes according to AR and Lin28 status. (A) DFS and OS by AR status in HER2 and TNBC breast cancer. There was no significant difference in survival between different AR statuses. (B) DFS and OS by Lin28 status in HER2 and TNBC breast cancer. There was no significant difference in survival between different Lin28 statuses.

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Availability of data and materials

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

Authors' contributions

DS, WP, KM, AK, TL and PO prepared the manuscript. DS and PO treated the patients. NS and TC performed pathological examination and data collection. WP, KM performed data collection and the statistical analysis. PO provided the concept of the study and finalized the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study has been approved by the ethics committee of the Faculty of Medicine, Siriraj Hospital, Mahidol University (Bangkok, Thailand; COA no. Si733/2016).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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