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Androgenic pathway in triple negative invasive ductal tumors: Its correlation with tumor cell proliferation

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Triple negative breast cancer (TNBC) is defined by estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 negativity. Patients with TNBC frequently undergo an aggressive clinical course due to the unavailability of specific targeted therapies. Androgen receptor (AR) was reported to be expressed in up to 60% of TNBC cases but there have been controversies as to the roles of androgen signaling through AR in TNBC. Therefore, in this study, we analyzed the status of AR in combination with androgen synthesizing enzymes (5α-reductase type 1 (5αR1) and 17β-hydroxysteroid dehydrogenase type 5 (17βHSD5)] in order to further understand androgenic actions in TNBC. Androgen receptor, 5αR1, and 17βHSD5 were immunolocalized in a cohort of 203 TNBC patients from Thailand and Japan. We then correlated the findings with clinicopathological characteristics (age, stage, tumor diameter, lymph node invasion, metastatic spread, Ki-67 labeling index, disease-free survival, and overall survival) of the patients. Univariate analysis revealed that AR+/enzyme+ cases were associated with a significantly lower Ki-67 labeling index than AR−/enzyme− samples. Multivariate analysis indicated the presence of significant positive correlations between AR and enzyme status in tumor cells, and between tumor diameter, lymph node invasion, and distant metastasis. Significant negative correlations were also detected between Ki-67 labeling index and AR status ($P = 0.04$) or 5αR1 ($P < 0.001$). Cox proportional hazards analysis showed that Ki-67 labeling index and stage were the only factors predicting disease-free and overall survival of the patients, although univariate Kaplan–Meier analysis revealed AR/5αR1 negativity suggested a more adverse clinical course up to 80 months after surgery. These results suggest that the presence of androgen synthesizing pathways in addition to AR expression in tumor cells could confer a better clinical outcome through suppression of cell proliferation. (*Cancer Sci* 2013; 104: 639–646)

Breast cancer is the most common malignancy in women[(1)] and, although recent advances in clinical management have significantly improved the survival rates of the great majority of breast cancer patients,[(2)] one subtype, so-called triple negative breast cancer (TNBC) continues to be associated with an adverse prognosis.[(3)] Triple negative breast cancer is characterized by the absence of estrogen receptor-α (ERα), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) expression in the tumor cells and constitutes approximately 6–60% of all breast cancer cases, depending on the cohort evaluated.[(4–23)] This subtype is considered to be far more diverse compared to other subtypes of breast malignancy.[(24–26)] Triple negative breast cancer is generally associated with relatively adverse clinical outcome[(27–30)] primarily due to the lack of specific therapies, higher rates of tumor cell proliferation, and more aggressive behavior.[(31)]

Numerous published studies have attempted to identify biomarkers that could further subclassify TNBC into disease subtypes. For instance, growth factors such as epidermal growth factor receptor[(32)] and insulin-like growth factor-1[(33)] have been studied and higher expression was reported to be associated with adverse clinical outcomes in TNBC patients. Other biomarkers reported so far include Numb protein,[(34)] chromosomal instability,[(35)] EZHR,[(36)] and miR34b.[(37)] Among these markers, one of the most extensively investigated but also one of the most controversial is the androgen receptor (AR).

The AR in triple negative breast tumor cells has been reported to be expressed at a relatively lower rate than in other types of breast cancer or even in breast cancer as a whole (50–100% in non-subtype specific[(38–43)]; 0–53% in TNBC[(4–19,21,22,44,45)]). However, the mere presence of AR in even a subset of TNBC patients suggests the manipulation of androgenic pathways in tumor cells could serve as a therapeutic option, at least for some TNBC patients. In addition, the availability of AR targeted agents, developed primarily for the treatment of prostate cancer, potentially makes the manipulation of androgenic pathways in TNBC patients more appealing as such treatment could be incorporated into clinical practice with less difficulty compared to other modes of target-specific therapies. The use of such therapies, however, is dependent on a clear understanding of the role of androgenic pathways in the biological behavior of TNBC.

The biological roles of androgens in TNBC are in dispute. Immunohistochemical studies looking at the presence of AR in tumor cells have reported conflicting results in terms of the correlation between AR and clinical outcome; some studies indicated a survival advantage,[(11,46,47)] others showed no significant effects of AR expression in tumor cells on survival of the patients.[(20,43,48)] However it is also true that, in contrast to these clinical studies, results of *in vitro* investigations consistently showed that AR may replace ER and PR as a driver of tumor proliferation and growth in TNBC cell lines.[(48–57)] The suggestion that AR expression could be an adverse marker is partially supported by one clinical study using gene expression profiles rather than immunoreactivity to define AR+ TNBC using

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luminal androgen receptor (LAR) gene expression profiles.[56] Based on these findings, clinical trials of AR antagonist have been initiated,[58] but further investigations are considered necessary to establish the precise roles of AR in TNBC patients.

We previously reported that AR expression in tandem with the presence of androgen synthesizing enzymes could eventually determine the clinical outcome for breast cancer patients as a whole, regardless of their intrinsic subtypes.[59] Therefore, we examined AR and androgen metabolizing enzymes in whole tissue sections of a cohort of 203 triple negative surgical breast cancer specimens obtained from both Japanese and Thai cohorts in order to evaluate the influence or effects of AR status in tumor cells on the Ki-67 labeling index of tumor cells, as well as on overall survival (OS) and disease-free survival (DFS) of the patients, in order to further extrapolate our findings on the receptor and enzymes in this study.

Materials and Methods

Patient cohorts. Following approval from the relevant institutional review boards or ethical committees (Japan, Tohoku University School of Medicine ID: 2012-1-185; Thailand, Mahidol University, Faculty of Medicine Ramathibodi Hospital ID: 01-54-50), archival materials of TNBC patients were retrieved from the surgical pathology files. The status of triple negativity in tumor cells was confirmed by reviewing the ER/PR/HER2 stained slides based on American Society of Clinical Oncology/College of American Pathologists guidelines. From these cohorts, a total of 203 TNBC specimens with whole tissue availability (Japan, 86 cases; Thailand, 117 cases) were examined. Clinical data including patient age, stage, tumor diameter, lymph node involvement, and metastatic spread was available in both cohorts after review of the charts of individual patients.

Immunohistochemistry. Archival tissue blocks were serially sectioned at a thickness of 4 μM and placed on pre-cleaned glue-coated glass slides for immunohistochemistry (IHC). Immunostaining of serial tissue sections for AR, 5α-reductase type 1 (5αR1) and 17β-hydroxysteroid dehydrogenase type 5 (17βHSD5) was carried out as described previously.[59,60] In brief, antibodies against all three targets (AR, AR441 1:50 [Dako, Carpinteria, CA, USA]; 5αR1, 1:1000, provided by Dr D.W. Russell [University of Texas Southwestern Medical Center, Dallas, TX, USA]; 17βHSD5, NP6.G6.A6 1:200 [Sigma, St. Louis, MO, USA]) were used in conjunction with a streptavidin–biotin visualization method (Histofine kit; Nichirei, Tokyo, Japan). A control tissue (AR, prostate; 5αR1, liver; 17βHSD5, adrenal) was included in all runs of immunostaining in order to confirm the specificity of immunostaining. Ki-67 immunostaining was carried out as described previously.[59]

Evaluation of immunoreactivity. Immunoreactivity was evaluated as follows. In an evaluation of AR in tumor cells, immunoreactivity was assessed by the H score.[61] In brief, the H score was obtained by assessing immunointensity (on a scale of 0–3) and prevalence in 100 cells over five different areas throughout the tumor (scale, 0–300). Cytoplasmic (5αR1, 17βHSD5) immunoreactivity was evaluated using a semiquantitative scale (0–2) which divided tumor cells into categories: no immunoreactivity; 0.1–50% immunoreactivity; and 50.1–100% immunoreactivity.[60] All slides were counted three times in order to assess intra-observer variability and variation was found to be less than 10%. In addition, a subset of tissue slides was assessed by at least two of the authors (KM, YN, KT) in order to assess inter-observer variability and inter-observer differences. Variation for the H score was less than 12%, and for the enzyme score less than 5%. For Ki-67 more than 500 tumor cells were counted in each case at the sites of hot spots. Hot spots were identified after evaluating the stained slides at low magnification. The immunoreactivity was quantified using a labeling index (LI). All the results were assessed by two authors (TY, HS) in order to assess reproducibility of the data, and found to be in agreement. When dichotomous variables were needed we used a cut-off point of 10% LI (converted from the H score) for AR, a score of 2 (>50% positivity) in enzyme staining.

Statistical analysis. Statistical analyses were carried out using JMP software (JMP Pro 9.0.2; SAS Institute, Tokyo, Japan). To assess the overlap between enzymes and receptor expression, as well as significant differences between categorical variables by country, a χ^2-test was used. Differences in linear variables between two different cohorts were tested using Student's *t*-test. Analysis of the effect of AR and enzyme expression on the Ki-67 LI was tested by stratifying the groups by AR/5αR1 status and using ANOVA followed by the Tukey–Kramer HSD *post-hoc* test. Correlation analysis was carried out using the Pearson correlation in order to compare the correlations among AR, enzyme expression, and clinical and pathological markers. Interactions between receptor and enzyme status and survival were tested using a multivariate Cox proportional hazards model, and statistical significance of individual factors examined was tested using Kaplan–Meier survival analysis.

Results

Androgen receptor, 5αR1, and 17βHSD5 in TNBC tumor cells. Androgen receptor immunoreactivity was detected in the nuclei of tumor cells and 17βHSD5 and 5αR1 immunoreactivity was detected in the cytoplasm of tumor cells. Immunostaining in serial tissue sections indicated that in the great majority of the cases examined, AR, 5αR1, and 17βHSD5 immunoreactivity, where present, was detected in comparable areas (Fig. 1).

Differences in clinicopathological parameters between cohorts. In order to asses any possible differences between the two separate cohorts, χ^2 assessment of the distribution of values between the Thai and the Japanese cohorts was undertaken. The number of patients and the percentage of populations (brackets) are given in Table 1. Immunoreactivity of 17βHSD5, lymph metastasis, and distant metastasis did not vary between Japanese and Thai cases but other factors such as age, stage, Ki-67, and tumor diameter were significantly different.

Prevalence of AR, 5αR1, and 17βHSD5 in TNBC patients. In 203 TNBC cases examined in this study, positive AR immunoreactivity in the tumor cells (defined as >10% LI) was detected in 51 cases (25%). The immunoreactivity of 5αR1 and 17βHSD5 in breast cancer cases was greater than that of AR, with 71.7% of cancers positive for 5αR1 and 69.8% for 17βHSD5. There were significant differences between the Thai and Japanese cohorts in terms of AR positivity, AR H score, and 5αR1 score ($P < 0.03$) but not in 17βHSD5 score ($P = 0.61$). These results are summarized in Table 1.

Correlation between AR, enzymes, and clinicopathological factors in TNBC. Using the χ^2-test, the status of AR and 17βHSD5 in tumor cells was significantly correlated ($P = 0.001$) (either double negative or double positive), and also that of AR and 5αR1 ($P = 0.04$) in the whole cohort. A significant positive correlation was also detected between these two enzymes in the whole cohort ($P = 0.001$, 53% of samples showing the same enzymatic score, 70% when classified as negative or positive). In the whole cohort of patients, 40% were receptor and enzymes double negative (AR−/5αR2−/17βHSD5−) and 8% were receptor/enzymes double positive (AR+/5αR2+/17βHSD5+) using a cut-off value of 2 (>50% immunoreactivity) for the enzymes and 10% LI for AR.

Pearson's correlation was used to assess the strength (Pearson's Rho; Table 2, first line for each parameter), and the significance (*P*-value; Table 2, second line for each parameter, values in parentheses) of correlations between various histologi-

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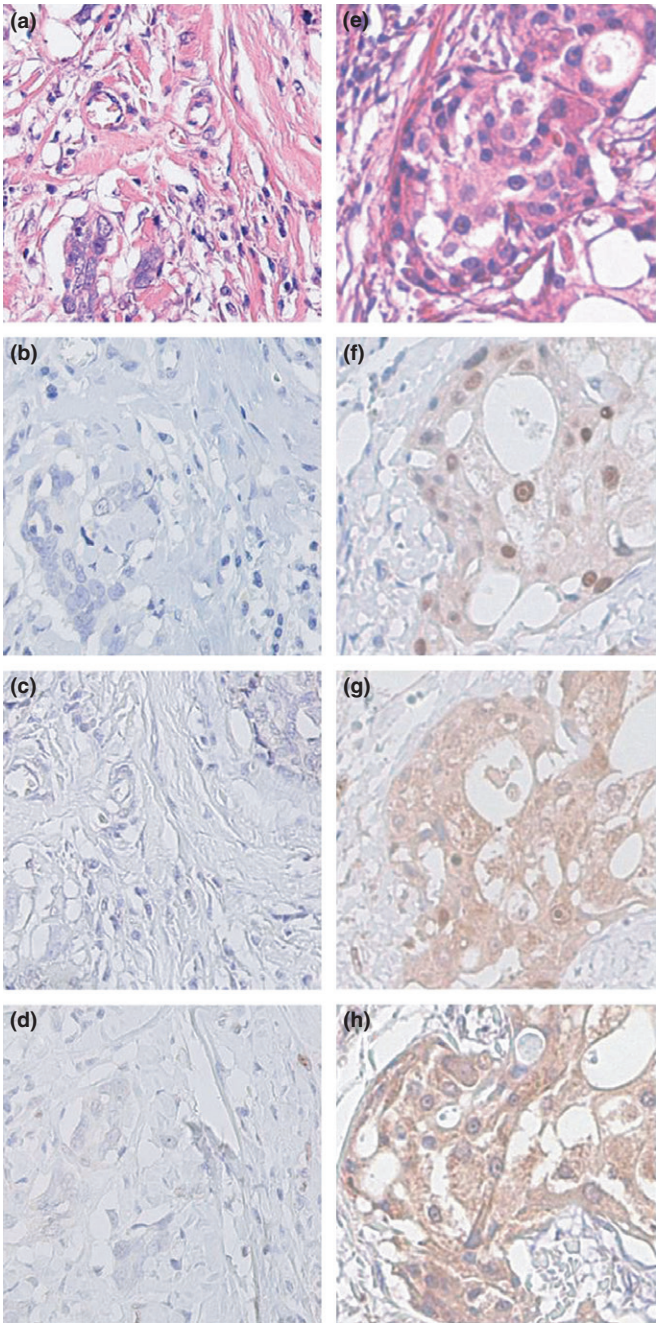

Fig. 1. Representative photographs of androgen receptor (AR), 5α-reductase type 1 (5αR1), and 17β-hydroxysteroid dehydrogenase type 5 (17βHSD5) immunohistochemistry in AR−5αR1−17βHSD5− (a–d) and AR+5αR1+17βHSD5+ (e–h) triple negative breast carcinomas. Hematoxylin–eosin staining (a,e). Androgen receptor was immunolocalized in the nuclei of carcinoma cells at variable immunoreactivity (b,f). Both 5αR1 (c,g) and 17βHSD5 (d,h) were immunolocalized in the cytoplasm of carcinoma cells. Stromal cells were negative in areas either adjacent or distal to carcinoma. Original magnification, x200.

cal and clinical parameters. Table 2 shows strong and significant (indicated in bold italics) or near significant (bold) correlations found between androgenic pathways as well as between androgenic pathways and indicators of tumor proliferation (AR and 5αR2, $P = 0.002$; AR and 17βHSD5, $P = 0.001$; AR and age, $P < 0.001$; 5αR1 and 17βHSD5, $P < 0.0001$), and also between the three clinicopathological factors used to define

Table 1. Summary of distribution of clinicopathological features in Thai, Japanese, and combined cohorts of patients with triple negative breast cancer (n = 203)

	Thai, *n* (%)	Japanese, *n* (%)	Combined	*P*-value
5αR1				
Negative	26 (22.2)	31 (36.4)	57 (28.2)	**0.03**
<50% positivity	28 (23.9)	23 (27.0)	51 (25.2)	
≥50% positivity	63 (53.8)	31 (36.4)	94 (46.5)	
17βHSD5				
Negative	34 (29.1)	27 (31.1)	61 (30.3)	0.61
<50% positivity	53 (45.2)	42 (48.8)	95 (46.7)	
≥50% positivity	30 (25.6)	17 (19.7)	47 (23.1)	
AR				
Positive (>10% LI)	20 (17.1)	31 (36.1)	51 (25.1)	0.002
Negative (≥10% LI)	97 (82.9)	55 (63.9)	152 (74.9)	
TNM stage				
I	32 (27.8)	30 (37.0)	62 (31.6)	**0.01**
IIA	46 (40.0)	27 (33.3)	73 (37.2)	
IIB	11 (9.6)	8 (9.9)	19 (9.7)	
IIIA	14 (12.2)	6 (7.4)	20 (10.2)	
IIIB	9 (7.8)	2 (2.4)	11 (5.6)	
IIIC	1 (0.9)	8 (9.9)	9 (4.6)	
IV	2 (1.7)	0 (0)	2 (1.0)	
Tumor size				
<20 mm	38 (33.9)	46 (56.7)	84 (43.5)	**0.005**
20.1–50 mm	63 (56.2)	31 (38.3)	94 (48.7)	
>50.1 mm	11 (9.8)	4 (4.9)	15 (7.7)	
Lymph invasion				
No	74 (65.5)	50 (62.5)	124 (64.3)	0.62
Yes	39 (34.5)	30 (37.5)	69 (35.8)	
Presence of distant metastasis				
Yes	2 (1.7)	0 (0)	2 (1.0)	0.23
No	113 (98.3)	81 (100)	194 (99.0)	
Age				
<50 years	59 (50.4)	29 (33.3)	88 (43.1)	0.04
≥50 years	58 (49.6)	58 (66.7)	116 (56.9)	
Ki-67				
<25%	75 (63.6)	30 (37.0)	105 (52.8)	**0.003**
≥25%	43 (36.4)	51 (63.0)	94 (47.2)	

Bold indicates significant value. 5αR1, 5α-reductase type 1; 17βHSD5, 17β-hydroxysteroid dehydrogenase type 5; AR, androgen receptor; LI, labeling index.

TNM breast cancer stage (tumor diameter, lymph node invasion, and metastatic spread). Significant negative correlations were also detected between Ki-67 LI and AR ($P = 0.048$) or 5αR1 ($P = 0.004$) status in tumor cells. The AR status tended to be inversely correlated with tumor diameter but this correlation did not reach statistical significance ($P = 0.055$). When stratified by country, the correlation coefficients obtained showed similar trends but not all significant associations remained.

In order to further assess the effects of AR/enzyme action on Ki-67 LI in tumor cells in TNBC cases, we subclassified the cases according to the AR/5αR1 status and compared the Ki-67 LI among these different groups of TNBC patients. We did not include 17βHSD5 in this stratification because of the relatively small cohort, and the close correlation between 17βHSD5 and 5αR1. In this analysis AR+/5αR1+ cases had the lowest Ki-67 LI and AR−/5αR1− the highest Ki-67 LI. (Fig. 2). The tendency among the four different groups still remained even if the Thai and Japanese cohorts were analyzed

Table 2. Pearson's correlations between clinicopathological features and androgen receptor (AR), 5α-reductase type 1 (5αR1), and 17β-hydroxysteroid dehydrogenase type 5 (17βHSD5) expression in combined cohort of patients with triple negative breast cancer from Japan and Thailand (n = 203)

	5αR1 score	17βHSD5 score	Age	Ki-67	Tumor diameter	Lymph node metastasis	Presence of distant metastasis
AR H score	***0.2151 (0.0020)***	***0.2438 (0.0010)***	***0.2948 (<0.0001)***	***–0.1414 (0.0480)***	**–0.1390 (0.0550)**	0.0920 (0.2040)	–0.0190 (0.7890)
5αR1 score		***0.4062 (<0.0010)***	0.0511 (0.4470)	***–0.2037 (0.0040)***	0.0405 (0.5780)	0.0343 (0.6380)	–0.0845 (0.2420)
17βHSD5 score			0.0740 (0.3010)	–0.0546 (0.4460)	0.0210 (0.7730)	–0.0292 (0.6870)	–0.0606 (0.4000)
Age				–0.0665 (0.3530)	–0.1274 (0.0780)	0.0319 (0.6600)	–0.1104 (0.1230)
Ki-67					–0.0205 (0.7780)	–0.0272 (0.7080)	0.0005 (0.9950)
Tumor diameter						***0.2808 (<0.0001)***	***0.1492 (0.0400)***
Lymph node invasion							**0.1395 (0.0540)**

Correlation strength was calculated using Pearson's Rho; significance (P-values) shown in parentheses. Bold, near significant correlation; bold italics, significant correlation.

separately, but only the Japanese cohort showed significance (P = 0.002) in separate analysis.

Analysis of the effects of AR and enzyme expression on DFS and OS. Interactions between AR, enzyme expression, and clinicopathological factors were modelled using a multivariate Cox proportional hazards model (Table 3). For OS, a total of 176 patients had complete data with 31 events; for DFS, a total of 174 patients were available with complete data with 35 events. For linear factors the risk ratio represents the change in risk per unit of the regressor, for ordinal factors the risk ratio is standardized to the lowest (i.e., in the least developed stage) grouping. In the stratification by country, the risk ratios were standardized to the Japanese cohort. Both models were significant predictors of outcome (OS, $P < 0.0001$; DFS, $P = 0.0005$). This analysis showed that the only robust and significant factors were tumor TNM stage at the time of diagnosis (which accounts for tumor diameter, lymph node involvement, and presence of distant metastasis), and Ki-67 LI ($P < 0.001$ and $P = 0.017$ respectively).

Results of univariate Kaplan–Meier analysis showed no significant effects on OS or DFS in evaluating AR+5αR1+ groups compared to others, but a significantly worse ($P < 0.05$) survival outcome was detected in AR-5αR1− patients in an 80-month follow-up period, which corresponds to the longest length of follow-up for patients in the Thai cohort (Fig. 3). The Ki-67 LI also predicted survival in the TNBC group in this analysis (Fig. 3).

Discussion

This study was undertaken to address controversies regarding the possible roles of androgenic pathways in the biological behavior of TNBC patients. In addition, we also hypothesized that assessing enzyme status in combination with receptor status could illuminate why contradictory results existed regarding the roles of androgen signalling in TNBC because of the biological importance of the intratumoral production of active steroids *in situ* (intracrinology).[62] In particular, we have previously shown that only the combination of the enzyme and AR expression (i.e., an intact androgen synthesis and signalling pathway) in tumor cells could predict a better clinical outcome for AR+ breast cancer patients, whereas AR status in tumor cells alone

Fig. 2. Correlation between androgen receptor (AR) H score and Ki-67 labeling index (LI) and effects on Ki-67 LI when triple negative breast cancer cases were subdivided according to receptor and enzyme status. The AR+ 5α-reductase type 1 (5αR1)+ group was significantly different from the AR−5αR1− group (P = 0.004, Tukey–Kramer HSD), based on the cut-off points >50% immunopositivity in 5αR1 and >10% labeling index for AR. If these results were stratified by country of origin, only the Japanese cohorts showed statistically significant differences. Thai cohorts showed a similar trend but the correlation did not reach statistical significance. The circles above the columns represent outlying points.

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Table 3. Interactions between androgenic pathways and clinical factors in determining survival outcomes in triple negative breast cancer patients from Thailand and Japan (n = 203)

	Overall survival				Disease-free survival			
	P-value	Risk ratio	CI lower	CI upper	*P-value*	Risk ratio	CI lower	CI upper
Ki-67	**0.0175**	6.80	1.42	40.75	**0.0404**	4.798	1.07	22.45
TNM stage								
IIa	**<0.0001**	0.81	0.24	2.86	**<0.0001**	1.18	0.42	3.59
IIb		5.17	1.37	20.39		3.72	0.99	13.42
IIIa		5.86	1.30	25.32		5.19	1.42	18.33
IIIb		3.61	0.17	26.59		3.95	0.55	19.15
IIIc		6.95	1.98	25.48		6.99	2.08	23.64
IV		401.97	34.79	4646.53		98.81	11.40	658.97
Country	0.8429	0.88	0.23	3.03	0.3792	0.64	0.23	1.72
AR H score	0.4268	1.00	0.99	1.01	0.7099	1.00	0.99	1.01
5αR1 score								
1	0.5886	1.54	0.48	4.89	0.6304	1.47	0.52	4.17
2		0.93	0.31	2.88		0.97	0.35	2.66
17βHSD5 score								
1	0.8235	0.75	0.29	1.99	0.6275	0.67	0.29	1.58
2		0.97	0.29	3.04		0.93	0.29	2.67
Age	0.4145	2.89	0.98	1.05	0.7953	1.00	0.97	1.04

Bold indicates significant value. 5αR1, 5α-reductase type 1; 17βHSD5, 17β-hydroxysteroid dehydrogenase type 5; AR, androgen receptor; CI, confidence interval.

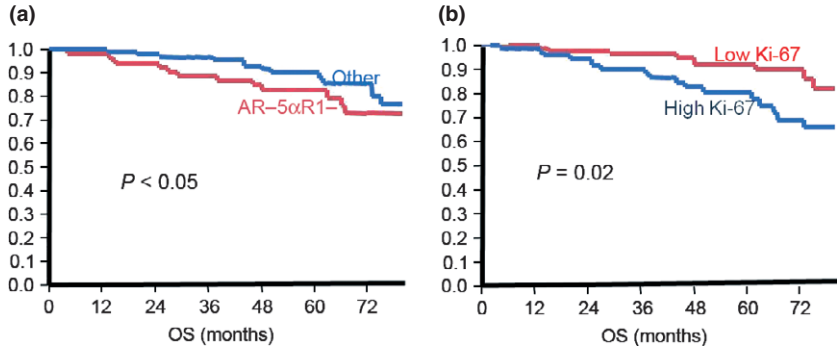

Fig. 3. Survival curves according to Ki-67 and androgen receptor (AR)−/5α-reductase type 1 (5αR1)− status of breast cancer patients. Kaplan–Meier survival curves analyzed according to 5αR1-/AR- (a) and Ki-67 labeling index (LI) (b). Double negative cases were tentatively defined as having less than 10% LI for AR and less than 50% of carcinoma areas immunohistochemically positive for 5αR1. Low Ki-67 cases were tentatively defined as less than 25% Ki-67 LI, with high Ki-67 cases greater than 25% LI (median value). Survival curves and analysis were truncated at 80 months because this was the longest time to follow up in the Thai cohort. If not truncated the survival curve of androgen action negative groups the patients crossed at approximately 90 months, but the Ki-67 survival curves did not cross until 150 months (the longest time of clinical follow-up in this study).

failed to indicate any significant effect of androgen signalling on prognosis of breast cancer patients.[59]

In our present study, all associations were tested by being stratified according to each cohort examined, as well as combined because of the differences in a number of clinicopathological factors between these two cohorts. This study was not designed to answer any possible cause of differences between the two cohorts, however, there are many factors we can speculate may be the cause, including but not limited to difference in ethnicity, and differences in methodological approaches between different hospitals. Despite these differences, the same trend was evident in terms of the possible correlation between various clinicopathological variables and those factors as related to androgenic signalling in tumor cells. Therefore, the underlying differences between these two cohorts are reasonably postulated not to hamper the validity of the conclusions in our study either when separated or combined.

Androgen receptor nuclear immunoreactivity was detected in approximately 25% of all TNBC cases examined, which fell into the ranges previously reported for TNBC.[45] In addition, AR immunoreactivity was associated with enzyme status, which is consistent with our previous findings in the whole series of breast cancer not stratified by individual subtypes.[59] The status of both AR and enzymes was independently associated with lower rates of Ki-67 LI of tumor cells and AR tended to be associated with a smaller tumor diameter, although the tendency did not reach statistical significance. These findings were also consistent with results of previously published studies regarding the correlation between AR and reduced Ki-67 LI in TNBC,[44,56] ER−,[23] and general breast cancer groups,[20] as well as the correlation between growth suppression in response to androgen therapy *in vivo*.[63,64] In addition, results of our present study indicated the presence of androgen synthesis enzymes in the tumors, which conferred an

additional contribution to decreased cell proliferation. It is also interesting to note that the lowest Ki-67 LI was indeed associated with AR+/5αR1+ cases followed by AR−/5aR1+, AR+/5aR1−, and AR−/5aR1− (Table 2). The statistically significant correlation between Ki-67 LI and survival was previously reported in TNBC cohorts,[65] which also suggests that AR and AR/enzyme expression may confer a survival advantage through the suppression of cell proliferation in TNBC.

In this study, it is also true that we could not show a significant effect of AR or enzyme expression on the overall clinical outcome of the patients, although the AR−5αR1− groups conferred an aggressive clinical course upon the patients examined in this study. One explanation for this finding could be that AR and androgen metabolism may not be the only factors responsible for cell proliferation, as in luminal A type breast cancer, but merely two of many governing cell proliferation. Hence, they may not necessarily be sufficient to significantly affect survival in our cohort, with its limited numbers of patients, especially in the AR+/enzyme+ group. The lack of correlation between AR and survival is not contradictory to published reports as, despite many studies showing a survival advantage associated with AR expression,[11,46,47] others have been unable to find a significant effect of AR expression on survival outcomes for patients.[20,43,48]

Results of the correlation analysis revealed that, aside from Ki-67, the only other clinical factor showing a statistically significant correlation with AR was patient age. This correlation has been detected in previous studies examining AR expression in breast cancer non- stratified by subtype,[7] ER− breast cancer,[46] and in TNBC,[56] although these results are not necessarily consistent.[19] A positive association between an increased level of circulating androgens in relation to estrogens and AR expression in the breast has been also reported in female to male transsexuals[66] and in prepubescent and postmenopausal primate breast tissue,[67] which suggest that correlations between age and AR expression may be explained by changes in the availability of circulating sex steroids. Further studies are needed to investigate what effect, if any, this increase in AR receptor expression with age may have on the underlying biology of TNBC. The lack of correlation between AR or enzyme expression and distant metastasis status or lymph node metastasis suggest that androgen signalling in TNBC cells might not play important roles in the process of tumor cell invasion and/or metastasis either to the lymphatic system or distant from the original tumor.

One of the major inconsistencies currently present in TNBC patients is the discrepancy between the majority of IHC results in TNBC cases and the results reported in AR+ TNBC cell lines. Although many clinical cohorts[11,46,47] and transient transfection[63] studies have shown clinically beneficial effects of AR expression in tumor cells, the great majority of AR+ TNBC cell lines, with the exception of MFM223 cells,[64] show growth stimulation or proliferation responses to androgen treatment,[57,68] mediated through activation of ER target genes[54] via androgen receptor actions.[48,52–55] Results of these *in vivo* studies suggest that AR expression in triple negative disease would be detrimental for the patients. The results are also in agreement with gene expression studies selecting for androgen-overexpressing ER− tumors, which indicated adverse clinical outcomes in OS[69] and disease recurrence.[56] Clinically these inconsistencies have posed serious problems in terms of deciding whether androgen inhibition or stimulation would prove beneficial in TNBC patients. Some potential explanations for the contradictions could be as follows.

First, there are often-raised differences between results in cell lines and human tissue specimens, as in other malignancies.[70–72] The three most widely used AR+ TNBC cell lines have significant mutations associated with intracellular signalling that are not necessarily recapitulated in the majority of TNBC specimens.[56,70,73,74] In addition, the most widely characterized TNBC cell line, MBD-MB-453, carries recently discovered mutations in the AR that alter its promiscuity to other ligands.[75] At this point the frequency of the mutation in the TNBC cancer population is totally unknown.

A second possible explanation is that the effects of AR expression in TNBC may not be homogeneous, or that gene expression profiling and IHC data of individual cases may not be selecting the same populations of patients. Gene expression profiling was initially used to show that breast cancer can be subtyped into categories that have meaningful clinical outcomes based upon their gene expression profiles.[76,77] Subsequent studies using gene expression profiles found groupings within breast cancer that are defined by the expression of AR in the absence of ER. These groupings have been termed molecular apocrine and LAR.[54,56] The molecular apocrine subtype is defined by the lack of a complete luminal or basal gene signature (as defined by Perou)[78] in combination with increment in AR signalling, whereas the LAR subtype is defined by enrichment of hormonally regulated pathways. It should be noted that the molecular apocrine classification included both HER2/ERBB2 enriched cancers in addition to triple negative cancers, and the triple negativity in the complete LAR set was defined by gene expression levels rather than a direct assessment of IHC reactivity of ER/PR and HER2. In both analyses, although the gene expression profiles selected for subgroups contained high levels of AR,[56,69] not all AR-expressing tumors were included in the LAR and molecular apocrine gene expression profiles (see Farmer *et al.*, fig. 3A[69] and Lehmann *et al.*, fig. S11[56]). This suggests that gene expression profiling is not just selecting for AR-expressing tumors but a subset of AR-expressing tumors that have an underlying biological signature. Interestingly, the gene profiles of the AR-expressing and AR growth-stimulated triple negative cell lines mentioned above all corresponded to the LAR subtype,[56] suggesting the currently available AR-expressing triple negative cell lines available might not be representative of the full spectrum of AR-expressing TNBC. Therefore, it is important to note that AR immunoreactivity in TNBC specimens does not necessarily indicate that these cases correspond to the androgen-enriched (LAR, molecular apocrine) subtypes of TNBC. Therefore, the possibility for divergent effects of AR action in TNBC could account for the current apparent contradictions in published reports between gene expression profile studies and clinical pathology studies. Further research into potential sub-subtyping of AR-expressing TNBC, including assessment of additional steroid receptors known to activated by androgen derivatives such as ERβ, may help to clarify the underlying biology.

In conclusion, results of our present study suggest that AR and androgen signalling pathways within the tumor may be beneficial to the clinical outcome of TNBC breast cancer patients through the inhibition of cellular proliferation. If borne out by further investigations, this could provide a subset of TNBC patients access to more targeted therapies than those currently available.

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Disclosure Statement

The authors have no conflicts of interest.

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