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PRECLINICAL STUDY

Evaluation of phospho-histone H3 in Asian triple-negative breast cancer using multiplex immunofluorescence

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Abstract

Purpose We used multiplex immunofluorescence (mIF) to determine whether mitotic rate represents an independent prognostic marker in triple-negative breast cancer (TNBC). Secondary aims were to confirm the prognostic significance of immune cells in TNBC, and to investigate the relationship between immune cells and proliferating tumour cells.

Methods A retrospective Asian cohort of 298 patients with TNBC diagnosed from 2003 to 2015 at the Singapore General Hospital was used in the present study. Formalin-fixed, paraffin-embedded breast cancer samples were analysed on tissue microarrays using mIF, which combined phospho-histone H3 (pHH3) expression with cytokeratin (CK) and leukocyte common antigen (CD45) expression to identify tumour and immune cells, respectively.

Results Multivariate analysis showed that a high pHH3 index was associated with significantly improved overall survival (OS; $p=0.004$), but this was not significantly associated with disease-free survival (DFS; $p=0.22$). Similarly, multivariate analysis also revealed that a pHH3 positive count of >1 cell per high-power field in the malignant epithelial compartment was an independent favourable prognostic marker for OS ($p=0.033$) but not for DFS ($p=0.250$). Furthermore, a high CD45 index was an independent favourable prognostic marker for DFS ($p=0.018$), and there was a significant positive correlation between CD45 and pHH3 index (Spearman rank correlation coefficient, 0.250; $p<0.001$).

Conclusions Mitotic rates as determined by pHH3 expression in epithelial cells are significantly associated with improved survival in TNBC. mIF analysis of pHH3 in combination with CK and CD45 could help clinicians in prognosticating patients with TNBC.

Keywords pHH3 · TNBC · Immune · Proliferation · Prognosis · Breast cancer

Abbreviations

ALN	Axillary lymph node
CD45	Leukocyte common antigen
CK	Cytokeratin
DFS	Disease-free survival
ER	Oestrogen receptor
FFPE	Formalin-fixed, paraffin-embedded
HER2	Human epidermal growth factor receptor 2

Chi Peng Timothy Lai, Joe Poh Sheng Yeong and An Sen Tan contributed equally to this work.

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mIF	Multiplex immunofluorescence
OS	Overall survival
pHH3	Phospho-histone H3
PR	Progesterone receptor
TILs	Tumour-infiltrating lymphocytes
TMA	Tissue microarray
TNBC	Triple-negative breast cancer

Introduction

Triple-negative breast cancer (TNBC), which is characterized by the lack of oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) expression [1], is a heterogeneous disease. While TNBC represents 10–24% of all breast cancer diagnoses globally, this varies with both geographic location and racial distribution [2]. In Singapore, TNBC accounts for 11% of breast cancers [3]. TNBC is typically associated with disproportionately high mortality rates, which result from its aggressive nature and a general lack of targeted therapies [4–6]. However, certain genomic sub-groups have a relatively favourable prognosis, further highlighting the heterogeneous nature of this disease [7]. To advance the development of appropriate systemic measures for patients with more favourable prognoses, novel prognostic agents that reliably stratify patients with TNBC are urgently required.

Recently, the relationship between immune infiltrates in tumour tissues and cancer has attracted substantial interest. TNBC has a robust immune microenvironment, with a greater abundance of tumour-infiltrating lymphocytes (TILs) compared with other breast cancers [4]. Furthermore, TILs serve as an independent prognostic marker in TNBC, with higher concentrations of TILs being linked with better prognosis [5]. However, the relationship between TILs and breast cancer prognosis is not straightforward and may be confounded by tumour proliferation rate. In a seminal study, Aaltomaa et al. [6] noted that the effectiveness of TIL density as a prognostic marker in breast cancer is limited to highly proliferative tumours only. This finding was further corroborated by a recent study by Nagalla et al. [7], which found that even under genetic analysis, the prognostic strength of immune metagenes in breast cancer was restricted to tumours within the highest proliferation tertile. However, despite the apparent association between tumour proliferation and the immune microenvironment, this relationship in breast cancers has not been widely studied.

Phospho-histone H3 (pHH3) is the phosphorylated state of histone H3, which is a core histone protein and a major component of chromatin [8]. Immunohistochemical detection of pHH3 can determine mitotic rate: phosphorylation of histone H3 begins in late G2 phase and is completed in late prophase [9], with dephosphorylation starting at the beginning of telophase [10]. Therefore, pHH3 can be seen as a surrogate marker for mitotic rate [11–13]. pHH3 has also shown promise as a prognostic marker in multiple types of cancer, including meningeal tumours [14], melanomas [11], and prostate cancers [15]. Furthermore, pHH3 is of prognostic significance in breast cancer, with a high-pHH3 count being associated with a relatively poor outcome [16, 17]. Although the role of pHH3 in breast cancers in general is well-studied, no previous studies have examined the prognostic significance of pHH3 in TNBCs.

Considering the dearth of information regarding pHH3 in TNBCs, as well as the uncertainty surrounding the relationship between tumour proliferation and the immune microenvironment, we aimed to investigate the significance of pHH3 as a prognostic marker in Asian TNBCs, as well as to ascertain the relationship between tumour proliferation and the immune microenvironment in TNBC. Using multimodal techniques including conventional pathology, multiplex immunofluorescence (mIF), and computer-assisted Vectra analysis, we retrospectively correlated clinicopathological parameters with pHH3, cytokeratin (CK; an epithelial cell marker) and leukocyte common antigen (CD45; a TIL marker) protein expression in an Asian cohort. Computer-assisted Vectra analysis was used to minimize inter-observer variability, and mIF was used to simultaneously evaluate pHH3 and CK expression for the quantification of tumour cell proliferation.

Methods and materials

Patients and tumours

A total of 406 patients with TNBC, diagnosed from 2003 to 2015 at the Department of Anatomical Pathology, Division of Pathology, Singapore General Hospital (SGH), were originally included in the present study. There was no follow-up data for 108 (26.6%) patients, and these patients were subsequently excluded from the study, leaving a final cohort of 298 patients. The median patient age at diagnosis was 55 years (range 28–89 years). Patients received adjuvant chemotherapy as per standard oncologic protocols for TNBC. Clinicopathological parameters, including age at diagnosis, tumour size, histological grade, histological subtype and axillary lymph node status were reviewed. Tumour subtypes and further details are summarised in Supplementary Table 1. Tumours and receptor status were characterised according to the World Health Organization and American Society of Clinical Oncology-College of American Pathologists (ASCO-CAP) guidelines [18].

Median follow-up was 43.8 months, ranging from 0.2 to 154.0 months, with a mean of 60.3 months. Disease recurrence occurred in 87 (21.4%) patients, and breast

cancer-associated mortality was documented in 51 (12.6%) patients across the cohort. In total, 65 (16.0%) patients succumbed to disease.

Tissue microarray (TMA) construction

Histological slides were retrieved and reviewed. Two representative tumour areas from each formalin-fixed, paraffin-embedded (FFPE) tissue block were identified, and a 1-mm diameter core obtained from each area. These tissue cores were subsequently assembled into TMAs using a Beecher microarrayer (Beecher Instruments, Inc., Sun Prairie, WI, USA).

Multiplex immunofluorescence (mIF)

mIF was performed using an Opal Multiplex fIHC kit (PerkinElmer, Inc., Waltham, MA, USA), as previously described by our group and in other studies [19–29]. FFPE tissue sections were processed according to a standard immunohistochemistry protocol, as previously described [30, 31]. Slides were labelled with primary antibodies against pHH3, CK and CD45, followed by appropriate secondary antibodies. Antibody details are presented in Supplementary Table 2. A fluorophore-conjugated tyramide signal amplification buffer (PerkinElmer, Inc.) was subsequently applied, and DAPI was used as a nuclear counterstain. Images were acquired using a Vectra 3 pathology imaging system microscope (PerkinElmer, Inc.) and analysed using inForm software (version 2.4.1; PerkinElmer, Inc.) [20, 32, 33] (Fig. 1 Image acquired using Vectra 3 pathology imaging system microscope).

Validation, follow-up and statistical analysis

Follow-up data were obtained from medical records. Disease-free survival (DFS) and overall survival (OS) were defined as the time from diagnosis to recurrence or death/date of last follow-up, respectively. Statistical analysis was performed using RStudio 1.1.456 running R 3.5.0 (R-core Team, R Foundation for Statistical Computing, Vienna, Austria) [34, 35]. Data import and processing were supported by the packages openxlsx, ggplot2, dplyr, tidyr, stringr, and survminer [35–42]. Survival outcomes were estimated using Kaplan–Meier analysis, and groups were compared using log-rank statistics. Multivariate Cox regression was performed to evaluate the effect of pHH3 expression on survival, after adjusting for clinicopathological parameters that included patient age at the point of diagnosis, tumour grade and axillary lymph node (ALN) status.

Maximally selected rank statistics [43] were applied using the maxstat R package to find optimal cut-off points to predict survival outcomes. The pHH3 index was defined as the percentage of $pHH3^+$ tumour cell nuclei out of the total number of tumour cells per high-power field of 1000 µm × 750 µm. Similarly, the CD45 index was defined as the percentage of $CD45^+$ cells out of the total number of cells, regardless of localization per high-power field of 1000 µm × 750 µm. Each variable was analysed independently by applying a cut-off point that identified TMAs with either high or low expression. Kaplan–Meier survival analysis and multivariate cox regression were performed on this processed data, as outlined above. A summary of the ranges, means, medians, and cut-offs used for all the variables analysed in this study is presented in Supplementary Table 3. Spearman's rank correlation coefficient was calculated to determine the degree of correlation between the pHH3 index and the CD45 index. $p < 0.05$ was considered to indicate a statistically significant difference.

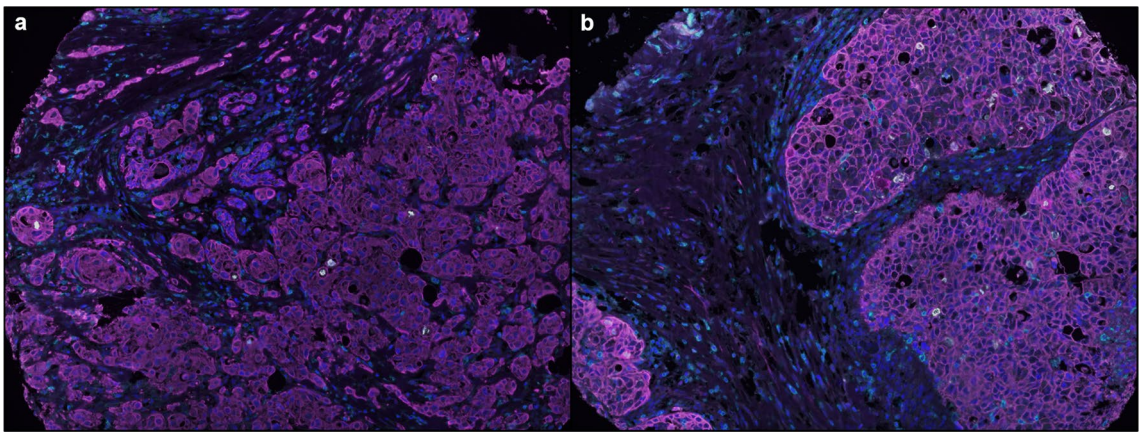

Fig. 1 Multiplex IF labelling sections from representative TNBC tissue samples. Multiplex IF labelling for PHH3 (white), CD45 (cyan), Pan-cytokeratin (magenta) and DAPI (dark blue). Representative multiplex IF staining showing **a** high and **b** low PHH3 expression in the epithelial compartment (magnification, 200x)

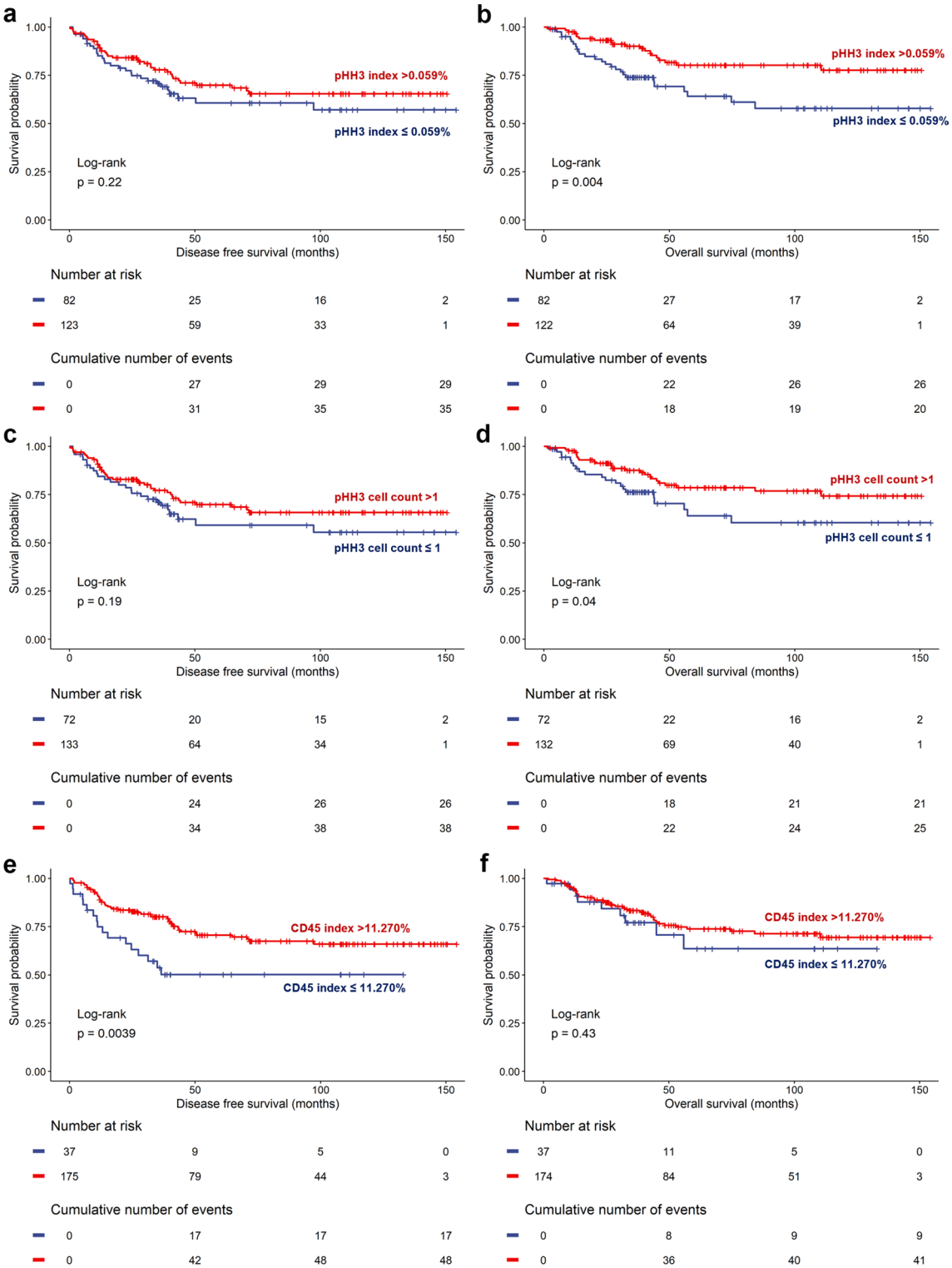

Fig. 2 High pHH3 expression is associated with improved survival in triple negative breast cancer. Kaplan-Meier analysis of **a** overall survival and **b** disease-free survival outcomes in women with high vs. low pHH3 index, Kaplan-Meier analysis of **c** overall survival and **d** disease-free survival outcomes in women with high vs. low pHH3 cell count per high-power field in the epithelial compartment, Kaplan-Meier analysis of **e** overall survival and **f** disease-free survival outcomes in women with high vs. low CD45 index. pHH3, phospho-Histone H3; CD45, leukocyte common antigen

Results

pHH3 index is associated with improved OS

Kaplan–Meier survival analysis revealed that a pHH3 index > 0.059% was not significantly associated with DFS (Log-rank $p = 0.22$; Fig. 2a Kaplan–Meier survival analysis for DFS using pHH3 index), but the pHH3 index was associated with significantly improved OS (Log-rank, $p = 0.004$; Fig. 2b Kaplan–Meier survival analysis for OS using pHH3 index). Multivariate analysis adjusted for tumour grade, age at diagnosis, and ALN status revealed that the pHH3 index was associated with favourable outcomes for OS (HR 0.477; 95% CI 0.252–0.902, $p = 0.023$). Positive ALN status was associated with reduced OS, while a higher tumour grade was associated with increased OS. Patient age at the point of diagnosis did not influence outcome (Table 1). However, while the pHH3 index was not significantly associated with DFS (HR 0.818; 95% CI 0.477–1.404, $p = 0.466$), increased ALN status was associated with reduced DFS (Table 1).

pHH3$^+$ cell count per high-power field is associated with improved OS in TNBC

Since a pHH3 index cut-off of 0.059% may not be practical in routine clinical practice, we also investigated pHH3$^+$ cell count per high-power field, which may be more amenable for routine application. While Kaplan–Meier survival analysis revealed that a pHH3$^+$ cell count of > 1 cell per high-power field (1000 µm × 750 µm) within the malignant epithelial compartment was not significantly associated with DFS (Log-rank, $p = 0.19$; Fig. 2c Kaplan–Meier survival analysis for DFS using pHH3 cell count in the epithelial compartment), it revealed that it was associated with significantly increased OS (Log-rank, $p = 0.04$; Fig. 2d Kaplan–Meier survival analysis for OS using pHH3 cell count in the epithelial compartment).

Confirming these results, multivariate analysis adjusted for tumour grade, age at diagnosis and axillary lymph node status revealed that a pHH3$^+$ cell count of > 1 cell per high-power field in the malignant epithelial compartment was an independent marker for favourable OS (HR 0.496; 95% CI 0.260–0.947, $p = 0.033$; Table 2) but not DFS (HR 0.722; 95% CI 0.415–1.257, $p = 0.250$; Table 2).

CD45 index is associated with improved DFS

CD45 was used as a surrogate for TILs. Kaplan–Meier survival analysis showed that a CD45 index > 11.270% was associated with significantly increased DFS (Log-rank, $p = 0.0039$; Fig. 2e Kaplan–Meier survival analysis for DFS using CD45 index) but not OS (Log-rank $p = 0.43$; Fig. 2f Kaplan–Meier survival analysis for OS using CD45 index). Multivariate analysis adjusted for tumour grade, age at diagnosis, and ALN status revealed that a high CD45 index was independently associated with favourable outcomes for DFS (HR 0.460; 95% CI 0.242–0.874, $p = 0.018$; Table 3) but not OS (HR 0.821;

Table 1 Multivariate Cox regression for overall survival and disease-free survival using pHH3 protein expression and clinicopathological parameters

Clinicopathological parameters	Overall survival		Disease-free survival	
	HR (95% CI)	*p* value	HR (95% CI)	*p* value
ALN status				
N0	Reference		Reference	
N1	3.121 (1.329–7.329)	0.009[a]	2.414 (1.243–4.689)	0.009[a]
N2	5.608 (2.478–12.693)	< 0.001[a]	3.160 (1.535–6.503)	0.002[a]
N3	34.495 (11.595–102.625)	< 0.001[a]	13.471 (5.276–34.399)	< 0.001[a]
Grade				
I	Reference		Reference	
II	0.094 (0.011–0.788)	0.029[a]	0.782 (0.085–7.193)	0.828
III	0.277 (0.057–1.354)	0.113	1.018 (0.131–7.936)	0.986
Age at diagnosis				
Age, years	0.981 (0.951–1.011)	0.216	0.986 (0.961–1.013)	0.301
pHH3 index (with cut-off = 0.059%)				
Low	Reference		Reference	
High	0.477 (0.252–0.902)	0.023[a]	0.818 (0.477–1.404)	0.466
	Adjusted for CD45 index:		Adjusted for CD45 index:	
	0.427 (0.206–0.882)	0.022[a]	0.787 (0.436–1.423)	0.429

ALN axillary lymph node status, *pHH3* phospho-histone H3

[a] $p < 0.05$ was considered to indicate a statistically significant difference

Table 2 Multivariate analysis of survival outcomes using pHH3 cell count in the epithelial compartment

Clinicopathological parameters	Overall survival		Disease-free survival	
	HR (95% CI)	*p* value	HR (95% CI)	*p* value
ALN status				
N0	Reference		Reference	
N1	3.115 (1.327–7.314)	0.009[a]	2.456 (1.262–4.776)	0.008[a]
N2	6.375 (2.759–14.731)	< 0.001[a]	3.378 (1.621–7.041)	0.001[a]
N3	37.451 (12.489–112.311)	< 0.001[a]	14.060 (5.470–36.140)	< 0.001[a]
Grade				
I	Reference		Reference	
II	0.097 (0.012–0.818)	0.032[a]	0.784 (0.085–7.217)	0.830
III	0.307 (0.064–1.473)	0.140	0.998 (0.128–7.765)	0.999
Age at diagnosis				
Age, years	0.980 (0.951–1.010)	0.198	0.985 (0.959–1.011)	0.257
pHH3 cell count per high-power field				
≤ 1	Reference		Reference	
> 1	0.496 (0.260–0.947)	0.033[a]	0.722 (0.415–1.257)	0.250
	Adjusted for CD45 index: 0.476 (0.238–0.952)	0.036[a]	Adjusted for CD45 index: 0.682 (0.382–1.219)	0.197

pHH3 phospho-histone H3

[a] $p < 0.05$ was considered to indicate a statistically significant difference

Table 3 Multivariate analysis of survival outcomes using CD45 index

Clinicopathological parameters	Overall survival		Disease-free survival	
	HR (95% CI)	*p* value	HR (95% CI)	*p* value
ALN status				
N0	Reference		Reference	
N1	2.581 (1.131–5.889)	0.024[a]	2.959 (1.525–5.744)	0.001[a]
N2	5.461 (2.455–12.150)	< 0.001[a]	3.378 (1.567–7.278)	0.002[a]
N3	23.523 (8.739–63.315)	< 0.001[a]	12.415 (5.141–29.985)	< 0.001[a]
Grade				
I	Reference		Reference	
II	0.231 (0.037–1.448)	0.118	1.040 (0.119–9.111)	0.972
III	0.528 (0.116–2.400)	0.409	1.168 (0.152–8.966)	0.882
Age at diagnosis				
Age, years	0.991 (0.963–1.012)	0.521	0.985 (0.959–1.011)	0.245
CD45 index (with cut-off = 11.270%)				
Low	Reference		Reference	
High	0.821 (0.352–1.917)	0.649	0.460 (0.242–0.874)	0.018[a]
	Adjusted for pHH3 index: 0.794 (0.315–2.001)	0.625	Adjusted for pHH3 index: 0.476 (0.240–0.945)	0.034

CD45 leukocyte common antigen

[a] $p < 0.05$ was considered to indicate a statistically significant difference

95% CI 0.352–1.917, $p = 0.649$; Table 3). Correlation analysis of the pHH3 index and the CD45 index is presented in Table 4, revealing a significant weak but positive correlation between the two, with Spearman rank correlation coefficient values of 0.250 (95% CI 0.1154–0.3832, $p < 0.001$; Table 4).

Prognostic value of pHH3 is independent of CD45 index

Although there was a weak correlation between pHH3 index and the CD45 index, the prognostic value of pHH3 was found to be independent of the number of TILs, measured

Table 4 Spearman rank correlation analysis between the pHH3 index and CD45 index

Parameter	PHH3 index versus CD45 index
Spearman correlation coefficient	0.250
95% CI	0.114–0.394
p value (two-tailed) for significance of correlation	<0.001[a]

pHH3 phospho-histone H3, *CD45* leukocyte common antigen

[a]$p<0.05$ was considered to indicate a statistically significant difference

by the CD45 index. Further multivariate analysis was performed adjusting for tumour grade, age at diagnosis, ALN status, and the CD45 index. The pHH3 index was significantly associated with OS even after adjusting for the CD45 index (HR 0.427; 95% CI 0.206–0.882; $p=0.022$; Table 1). Similarly, the pHH3 cell count in the epithelial compartment per high-power field was associated with significantly increased OS (HR 0.476; 95% CI 0.238–0.952; $p=0.036$; Table 2). No significant association was found between either variable and DFS, as with the initial multivariate analyses.

Prognostic value of CD45 is independent of pHH3 index

Although there was a weak correlation between pHH3 index and the CD45 index, the prognostic value of CD45 was found to be independent of tumour proliferation, measured by the pHH3 index. Further multivariate analysis was performed adjusting for tumour grade, age at diagnosis, ALN status, and the pHH3 index. The CD45 index was significantly associated with DFS even after adjusting for the pHH3 index (HR 0.476; 95% CI 0.240–0.945; $p=0.034$; Table 3). No significant association was found between either variable and OS, as with the initial multivariate analyses.

Discussion

The major conclusion of the present study is that elevated pHH3 expression is associated with favourable outcomes in TNBC, characterized by increased OS. Through our large patient cohort, we demonstrated that the prognostic significance of pHH3 in TNBC is independent of age, tumour grade, ALN status, and CD45 index. To the best of our knowledge, this study is the first to show that pHH3 expression has prognostic significance in TNBC.

Highly proliferative breast cancers are known to be associated with an improved response to chemotherapy [44, 45]. Sillem et al. [46] previously found that an increased pHH3 count in breast cancer was linked to improved pathological response. However, even though breast cancers with high-pHH3 expression have an improved response to chemotherapy, the overall outcome has previously been found to be worse compared with less-proliferative tumours with low pHH3 expression [16, 17, 47–50]. This may be because a high degree of proliferation is typically indicative of tumour aggression, and on balance this aggression may outweigh the effect of increased chemosensitivity.

The results of the present study appear to contradict those of previous studies, as the data indicated that pHH3 count in tumour cells was associated with improved outcome, in the form of increased OS. This contradiction can be attributed to the unique characteristics of TNBC. While TNBC is known to be aggressive [51–53] and to have a high-proliferation rate [51, 54], this subtype is also more sensitive to chemotherapy than other types of breast cancer [55–57]. We postulate that the inherent high-proliferation rate of TNBC confounds the effect of proliferation on outcome, such that the effect of increased chemosensitivity supersedes the associated aggressiveness, resulting in an improved outcome compared to patients with a low pHH3 count. We also note that the association between high pHH3 count and outcome was only significant for OS, not DFS. This is possibly because TNBCs with high pHH3 expression may recur quickly. TNBC is known to have an earlier peak of recurrence than other types of cancer [52, 58], and highly proliferative tumours also have increased rates of recurrence [59, 60]. In a previous study on patterns of breast cancer recurrence, Ribelles et al. [61] found that TNBC with a low-proliferation rate had a smooth risk curve, while TNBC with a high-proliferation rate had a sharp peak of recurrence at 18 months. Therefore, while the prognostic value of pHH3 in DFS may be confounded by the early pattern of recurrence shown by these highly proliferative TNBCs, the prognostic value of pHH3 is not compromised in OS as the recurring tumours are similarly highly proliferative and chemosensitive. The amenability to chemotherapy could contribute to the improved OS.

Immune infiltrates are known to serve a prognostic function in TNBC [5]. In line with previous studies, we found that the presence of a higher proportion of immune cells led to significantly improved DFS, although OS was not significantly improved. We postulate that the prognostic impact of immune infiltrates, as denoted by $CD45^+$ cells, on OS was not significant because the marker CD45 is expressed by a wide variety of immune cells [62–64]. There are many different types of immune cells in the tumour microenvironment, and these may have contrasting functions: while some immune cells eradicate malignant cells, others may enhance tumour growth [65]. Therefore, simply quantifying immune cells as a whole without specifying subtype or function is insufficient to predict OS, despite the apparent role in DFS.

Notably, to our best knowledge, the present study is the first to demonstrate a direct relationship between tumour proliferation and TIL density in Asian TNBC. Aaltomaa et al. [6] have previously shown that TIL density as a prognostic marker is limited to highly proliferative tumours only, but this was done in a Western cohort of breast cancer patients. There was a weak but significant positive relationship between tumour proliferation and TIL density in our cohort, suggesting that highly proliferative tumours are more immunogenic. We theorise that highly proliferative tumours, being associated with increased cell death [66–69], result in a greater release of tumour antigens. As a minimum antigen load is needed to trigger and potentiate a strong immune response [70–73], immune cells in the tumour microenvironment are only sufficiently activated to significantly affect prognosis when this threshold is reached. This could explain the observations by Aaltomaa et al. [6] and Nagalla et al. [7], who found that the prognostic power of immune cells was limited to highly proliferative tumours. However, given that the correlation found in our study was weak, further studies in other types of breast cancer are needed to establish the legitimacy of this relationship. One limitation of the present study is that only TNBCs were studied, which might not be the best candidates to analyse the relationship between tumour proliferation and immune infiltrates, given that the inherent high-proliferation rate [4, 5] and immunogenicity [4] of TNBCs may confound this relationship.

In our study, the prognostic value of CD45 was independent of tumour proliferation. This is in contrast to findings of Aaltomaa et al. [6] and Nagalla et al. [7], who determined that the prognostic value of CD45 was confounded by tumour proliferation.

The results of the present study may help in further defining the benefit of adjuvant chemotherapy in TNBC patients. Currently, adjuvant chemotherapy is recommended for tumours larger than 0.5 cm [74, 75]. Therapy in TNBC patients with high pHH3 expression could be modulated based on considerations of greater chemosensitivity.

While our study investigates TNBC patients who received adjuvant chemotherapy, the results may also apply to patients treated with neo-adjuvant therapy. Neo-adjuvant therapy is typically used for certain high-risk breast cancers, such as larger tumours and locally advanced disease that are initially ineligible for resection [76, 77]. pHH3 has been demonstrated to have predictive value in breast cancer patients treated with neo-adjuvant protocols, with high-pHH3 expression being associated with improved pathological complete response [46]. However, whether this improvement in pathological complete response translates into better OS remains to be seen. Further studies on cohorts of TNBC patients treated with neo-adjuvant therapies are needed to fully ascertain the role of pHH3 in TNBC, particularly in Asian populations.

One further potential application of the present study is the use of pHH3 in immunotherapy. Our findings suggest that highly proliferative tumours, as denoted by high pHH3 expression, are possibly more immunogenic and lend themselves to robust immune responses. Further studies should be performed to confirm this relationship, and to further elucidate the mechanisms through which tumour proliferation affects immune response. Results of such studies may be of significance for guiding future immunotherapeutic approaches in cancer, and may help identify patients best suited for immunotherapy. Patients with highly proliferative tumours could be considered for immunotherapy, as their tumours are more likely to respond.

Limitations

One limitation of our study is that this study is undertaken on TMA cores. One criticism of using TMA cores is that for each specimen only a small amount of tissue is sampled and arrayed, such that sampling error may lead to a reduced representation of the full tissue section. This limitation is especially pertinent in breast cancers, given that they display intra-tumour heterogeneity [78, 79]. Whilst we acknowledge that TMA cores are less ideal than whole sections, it is our view that there is enough concordance between TMA cores and whole sections for our results on TMA cores to be of significance. In a study on lymphocytic infiltrates in breast cancer, Khan et al. [80] observed a fair degree of concordance between core-based and tumour-based scores with only 1 TMA core (AUC = 0.90). As for the efficacy of TMA cores in evaluating proliferation, Tay et al. [81] used TMA cores to determine the optimal threshold for Ki67 (another proliferative marker similar to pHH3) predicting outcomes of invasive breast cancer—they found that the results translated into survival correlations and thresholds which mirrored those in other reported cohorts which used whole slides. Therefore, we conclude that TMA can be used as a surrogate tool for discovery research as reported here. However, we are aware that further validation with larger cohorts and whole slides is warranted.

Another limitation of our study is that we only characterised TILs by CD45 positivity, with no further immunophenotyping to define the immune subtype, as we have already highlighted the roles and prognostic significance of the different subtypes of immune cells in previous publications [23, 30, 82, 83]. In this study, we used CD45 to quantify the total effect of immune infiltrates on prognosis in Asian TNBCs.

Conclusion

In conclusion, the present study established that high pHH3 expression is associated with a positive outcome in TNBC. Our study also suggested the existence of a significant positive relationship between tumour proliferation and immune response in TNBC. As pHH3 can be accurately scored through computer-assisted image analysis [84, 85], a prognostic system involving this marker may be an effective way to select treatment options for patients with TNBC.

Author contributions PT and JY conceived and directed the study. PT, and JY supervised the research. JL constructed TMAs, performed IHC, prepared samples for NanoString, and collated data. BL performed bioinformatics analysis. AT, JY and TL performed immunohistochemical scoring, interpreted the data and performed biostatistical analysis. CO constructed TMAs, performed IHC, and collated data. TP, AT, JI, RD, and EL contributed to the scientific content of the study. AT, JY, and TL drafted the manuscript with the assistance and final approval of all authors.

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Availability of data and materials The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The SingHealth Centralized Institutional Review Board (CIRB) approved the authors' request for waiver of informed consent based on ethical consideration (Ref: 2011/433/F). The SingHealth CIRB operates in accordance with the ICH/Singapore Guideline for Good Clinical Practices, and with the applicable regulatory requirement(s). This article does not contain any studies with animals performed by any of the authors.

Informed consent The SingHealth Centralized Institutional Review Board (CIRB) approved the authors' request for waiver of informed consent based on ethical consideration (Ref: 2011/433/F). The SingHealth CIRB operates in accordance with the ICH/Singapore Guideline for Good Clinical Practices, and with the applicable regulatory requirement(s).

References

1. Aysola K, Desai A, Welch C, Xu J, Qin Y, Reddy V et al (2013) Triple negative breast cancer—an overview. Hered Genet: Curr Res 2013(Suppl 2):001
2. Plasilova ML, Hayse B, Killelea BK, Horowitz NR, Chagpar AB, Lannin DR (2016) Features of triple-negative breast cancer: analysis of 38,813 cases from the national cancer database. Medicine 95(35):e4614-e
3. Thike AA, Cheok PY, Jara-Lazaro AR, Tan B, Tan P, Tan PH (2010) Triple-negative breast cancer: clinicopathological characteristics and relationship with basal-like breast cancer. Mod Pathol 23:123–133
4. Jang N, Kwon HJ, Park MH, Kang SH, Bae YK (2018) Prognostic value of tumor-infiltrating lymphocyte density assessed using a standardized method based on molecular subtypes and adjuvant chemotherapy in invasive breast cancer. Ann Surg Oncol 25(4):937–946
5. Adams S, Gray RJ, Demaria S, Goldstein L, Perez EA, Shulman LN et al (2014) Prognostic value of tumor-infiltrating lymphocytes in triple-negative breast cancers from two phase III randomized adjuvant breast cancer trials: ECOG 2197 and ECOG 1199. J Clin Oncol 32(27):2959–2966
6. Aaltomaa S, Lipponen P, Eskelinen M, Kosma VM, Marin S, Alhava E et al (1992) Lymphocyte infiltrates as a prognostic variable in female breast cancer. Eur J Cancer 28(4):859–864
7. Nagalla S, Chou JW, Willingham MC, Ruiz J, Vaughn JP, Dubey P et al (2013) Interactions between immunity, proliferation and molecular subtype in breast cancer prognosis. Genome Biol 14(4):R34-R
8. Hendzel MJ, Wei Y, Mancini MA, Van Hooser A, Ranalli T, Brinkley BR et al (1997) Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. Chromosoma 106(6):348–360
9. Hans F, Dimitrov S (2001) Histone H3 phosphorylation and cell division. Oncogene 20(24):3021–3027
10. Olar A (2015) Phosphohistone H3: implications for clinical practice and risk assessment in meningioma. Neuro-oncology 17(5):631–633
11. Hale CS, Qian M, Ma MW, Scanlon P, Berman RS, Shapiro RL et al (2013) Mitotic rate in melanoma: prognostic value of immunostaining and computer-assisted image analysis. Am J Surg Pathol 37(6):882–889
12. Varughese RK, Lind-Landström T, Habberstad AH, Salvesen Ø, Haug CS, Sundstrøm S et al (2016) Mitosin and pHH3 predict poorer survival in astrocytomas WHO grades II and III. J Clin Pathol 69(1):26
13. Chow KL, Tse KY, Cheung CL, Wong KW, Cheung AN, Wong RW et al (2017) The mitosis-specific marker phosphohistone-H3 (PHH3) is an independent prognosticator in uterine smooth muscle tumours: an outcome-based study. Histopathology 70(5):746–755
14. Elmaci I, Altinoz MA, Sari R, Bolukbasi FH (2018) Phosphorylated histone H3 (PHH3) as a novel cell proliferation marker and prognosticator for meningeal tumors: a short review. Appl Immunohistochem Mol Morphol: AIMM 26(9):627–631
15. Goltz D, Montani M, Braun M, Perner S, Wernert N, Jung K et al (2015) Prognostic relevance of proliferation markers (Ki-67, PHH3) within the cross-relation of ERG translocation and androgen receptor expression in prostate cancer. Pathology 47(7):629–636
16. Skaland I, Janssen E, Gudlaugsson E, Klos J, Kjellevold KH, Søiland H et al (2007) Phosphohistone H3 expression has much stronger prognostic value than classical prognosticators

in invasive lymph node-negative breast cancer patients less than 55 years of age. Mod Pathol 20(12):1307–1315
17. Gerring Z, Pearson JF, Morrin HR, Robinson BA, Harris GC, Walker LC (2015) Phosphohistone H3 outperforms Ki67 as a marker of outcome for breast cancer patients. Histopathology 67(4):538–547
18. Lakhani S, Ellis I, Schnitt S, Tan P, Van de Vijver M (2012) World Health Organisation classification of tumors of the breast. Int Agency Res Cancer 4:142–147
19. Stack EC, Wang C, Roman KA, Hoyt CC (2014) Multiplexed immunohistochemistry, imaging, and quantitation: a review, with an assessment of Tyramide signal amplification, multispectral imaging and multiplex analysis. Methods 70(1):46–58
20. Abel EJ, Bauman TM, Weiker M, Shi F, Downs TM, Jarrard DF et al (2014) Analysis and validation of tissue biomarkers for renal cell carcinoma using automated high-throughput evaluation of protein expression. Hum Pathol 45(5):1092–1099
21. Lovisa S, LeBleu VS, Tampe B, Sugimoto H, Vadnagara K, Carstens JL et al (2015) Epithelial-to-mesenchymal transition induces cell cycle arrest and parenchymal damage in renal fibrosis. Nat Med 21(9):998–1009
22. Garnelo M, Tan A, Her Z, Yeong J, Lim CJ, Chen J et al (2015) Interaction between tumour-infiltrating B cells and T cells controls the progression of hepatocellular carcinoma. Gut 15(310814):2015–310814
23. Yeong J, Thike AA, Lim JC, Lee B, Li H, Wong SC et al (2017) Higher densities of Foxp3(+) regulatory T cells are associated with better prognosis in triple-negative breast cancer. Breast Cancer Res Treat 163(1):21–35
24. Garnelo M, Tan A, Her Z, Yeong J, Lim CJ, Chen J et al (2017) Interaction between tumour-infiltrating B cells and T cells controls the progression of hepatocellular carcinoma. Gut 66(2):342–351
25. Lim JCT, Yeong JPS, Lim CJ, Ong CCH, Chew VSP, Ahmed SS, Tan PH, Iqbal J (in press) An automated staining protocol for 7-colour immunofluorescence of human tissue sections for diagnostic and prognostic use. J R Coll Pathol Australas
26. Esbona K, Inman D, Saha S, Jeffery J, Schedin P, Wilke L et al (2016) COX-2 modulates mammary tumor progression in response to collagen density. Breast Cancer Res 18(1):35
27. Mlecnik B, Bindea G, Kirilovsky A, Angell HK, Obenauf AC, Tosolini M et al (2016) The tumor microenvironment and immunoscore are critical determinants of dissemination to distant metastasis. Sci Transl Med 8(327):327ra26
28. Nghiem PT, Bhatia S, Lipson EJ, Kudchadkar RR, Miller NJ, Annamalai L et al (2016) PD-1 blockade with pembrolizumab in advanced merkel-cell carcinoma. New Engl J Med 374(26):2542–2552
29. Feng Z, Jensen SM, Messenheimer DJ, Farhad M, Neuberger M, Bifulco CB et al (2016) Multispectral imaging of T and B cells in murine spleen and tumor. J Immunol 196(9):3943–3950
30. Yeong J, Lim JCT, Lee B, Li H, Chia N, Ong CCH et al (2018) High densities of tumor-associated plasma cells predict improved prognosis in triple negative breast cancer. Front Immunol 9:1209
31. Yeong J, Thike AA, Lim JC, Lee B, Li H, Wong SC et al (2017) Higher densities of Foxp3 + regulatory T cells are associated with better prognosis in triple-negative breast cancer. Breast Cancer Res Treat 23(10):017–4161
32. Fiore C, Bailey D, Conlon N, Wu X, Martin N, Fiorentino M et al (2012) Utility of multispectral imaging in automated quantitative scoring of immunohistochemistry. J Clin Pathol 65(6):496–502
33. Feng Z, Bethmann D, Kappler M, Ballesteros-Merino C, Eckert A, Bell RB et al (2017) Multiparametric immune profiling in HPV—oral squamous cell cancer. JCI Insight 2(14):e93652
34. RStudio (2015) RStudio: integrated development environment for R. RStudio Inc, Boston
35. R Foundation for Statistical Computing (2016) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna
36. Walker A. openxlsx: read, write and edit XLSX files. R package version 3.0.02015
37. Wickham H (2009) ggplot2: elegant graphics for data analysis. Springer, New York
38. Wickham H (2011) The split-apply-combine strategy for data analysis. J Stat Softw 40:1–29
39. Wickham H. tidyr: easily tidy data with `spread()` and `gather()` functions. R package version 0.6.02016
40. Wickham H. stringr: simple, consistent wrappers for common string operations. R package version 1.1.02016
41. Wickham H, Francois R. dplyr: a grammar of data manipulation. R package version 0.5.02016
42. Kassambara A, Kosinski M. survminer: drawing survival curves using 'ggplot2'. R package version 0.2.42016
43. Lausen B, Schumacher M (1992) Maximally selected rank statistics. Biometrics 48(1):73–85
44. Stover DG, Coloff JL, Barry WT, Brugge JS, Winer EP, Selfors LM (2016) The role of proliferation in determining response to neoadjuvant chemotherapy in breast cancer: a gene expression-based meta-analysis. Clin Cancer Res 22(24):6039–6050
45. Amadori D, Volpi A, Maltoni R, Nanni O, Amaducci L, Amadori A et al (1997) Cell proliferation as a predictor of response to chemotherapy in metastatic breast cancer: a prospective study. Breast Cancer Res Treat 43(1):7–14
46. Sillem M, Timme S, Bronsert P, Bogatyreva L, Hauschke D, Zur Hausen A et al (2017) Anti-Phosphohistone H3-positive mitoses are linked to pathological response in neoadjuvantly treated breast cancer. Breast Care 12(4):244–250
47. Klintman M, Strand C, Ahlin C, Beglerbegovic S, Fjallskog ML, Grabau D et al (2013) The prognostic value of mitotic activity index (MAI), phosphohistone H3 (PPH3), cyclin B1, cyclin A, and Ki67, alone and in combinations, in node-negative premenopausal breast cancer. PLoS ONE 8(12):e81902
48. Gudlaugsson E, Klos J, Skaland I, Janssen EA, Smaaland R, Feng W et al (2013) Prognostic comparison of the proliferation markers (mitotic activity index, phosphohistone H3, Ki67), steroid receptors, HER2, high molecular weight cytokeratins and classical prognostic factors in T(1)(-)(2)N(0)M(0) breast cancer. Pol J Pathol 64(1):1–8
49. Gudlaugsson E, Skaland I, Undersrud E, Janssen EA, Soiland H, Baak JP (2011) D2-40/p63 defined lymph vessel invasion has additional prognostic value in highly proliferating operable node negative breast cancer patients. Mod Pathol 24(4):502–511
50. Kim J-Y, Jeong HS, Chung T, Kim M, Lee JH, Jung WH et al (2017) The value of phosphohistone H3 as a proliferation marker for evaluating invasive breast cancers: a comparative study with Ki67. Oncotarget 8(39):65064–65076
51. Rhee J, Han S-W, Oh D-Y, Kim JH, Im S-A, Han W et al (2008) The clinicopathologic characteristics and prognostic significance of triple-negativity in node-negative breast cancer. BMC Cancer 8(1):307
52. Dent R, Trudeau M, Pritchard KI, Hanna WM, Kahn HK, Sawka CA et al (2007) Triple-negative breast cancer: clinical features and patterns of recurrence. Clin Cancer Res 13(15 Pt 1):4429–4434
53. Haffty BG, Yang Q, Reiss M, Kearney T, Higgins SA, Weidhaas J et al (2006) Locoregional relapse and distant metastasis in conservatively managed triple negative early-stage breast cancer. J Clin Oncol 24(36):5652–5657
54. Keam B, Im SA, Kim HJ, Oh DY, Kim JH, Lee SH et al (2007) Prognostic impact of clinicopathologic parameters in stage II/III breast cancer treated with neoadjuvant docetaxel and doxorubicin chemotherapy: paradoxical features of the triple negative breast cancer. BMC Cancer 7:203

55. Sirohi B, Arnedos M, Popat S, Ashley S, Nerurkar A, Walsh G et al (2008) Platinum-based chemotherapy in triple-negative breast cancer. Ann Oncol 19(11):1847–1852
56. Uhm JE, Park YH, Yi SY, Cho EY, Choi YL, Lee SJ et al (2008) Treatment outcomes and clinicopathologic characteristics of triple-negative breast cancer patients who received platinum-containing chemotherapy. Int J Cancer 124(6):1457–1462
57. Carey LA, Dees EC, Sawyer L, Gatti L, Moore DT, Collichio F et al (2007) The triple negative paradox: primary tumor chemosensitivity of breast cancer subtypes. Clin Cancer Res 13(8):2329
58. Liedtke C, Mazouni C, Hess KR, Andre F, Tordai A, Mejia JA et al (2008) Response to neoadjuvant therapy and long-term survival in patients with triple-negative breast cancer. J Clin Oncol 26(8):1275–1281
59. Lelotte J, Mourin A, Fomekong E, Michotte A, Raftopoulos C, Maiter D (2018) Both invasiveness and proliferation criteria predict recurrence of non-functioning pituitary macroadenomas after surgery: a retrospective analysis of a monocentric cohort of 120 patients. Eur J Endocrinol 178(3):237–246
60. Feng W, Malpica A, Skaland I, Gudlaugsson E, Robboy SJ, Dalen I et al (2013) Can proliferation biomarkers reliably predict recurrence in world health organization 2003 defined endometrial stromal sarcoma, low grade? PLoS ONE 8(10):e75899
61. Ribelles N, Perez-Villa L, Jerez JM, Pajares B, Vicioso L, Jimenez B et al (2013) Pattern of recurrence of early breast cancer is different according to intrinsic subtype and proliferation index. Breast Cancer Res 15(5):R98-R
62. Trowbridge IS (1978) Interspecies spleen-myeloma hybrid producing monoclonal antibodies against mouse lymphocyte surface glycoprotein, T200. J Exp Med 148(1):313–323
63. Michaelson J, Scheid M, Boyse EA (1979) Biochemical features of Ly-5 alloantigen. Immunogenetics 9(1):193–197
64. Scheid MP, Triglia D (1979) Further description of theLy-5 system. Immunogenetics 9(1):423–433
65. Johansson M, Denardo DG, Coussens LM (2008) Polarized immune responses differentially regulate cancer development. Immunol Rev 222:145–154
66. Bai M, Agnantis NJ, Kamina S, Demou A, Zagorianakou P, Katsaraki A et al (2001) In vivo cell kinetics in breast carcinogenesis. Breast Cancer Res 3(4):276–283
67. Lee JS, Kim HS, Jung JJ, Kim YB, Park CS, Lee MC (2001) Correlation between angiogenesis, apoptosis and cell proliferation in invasive ductal carcinoma of the breast and their relation to tumor behavior. Anal Quant Cytol Histol 23(2):161–168
68. Lipponen P (1999) Apoptosis in breast cancer: relationship with other pathological parameters. Endocr Relat Cancer 6(1):13–16
69. Schulte-Hermann R, Bursch W, Grasl-Kraupp B, Marian B, Torok L, Kahl-Rainer P et al (1997) Concepts of cell death and application to carcinogenesis. Toxicol Pathol 25(1):89–93
70. Munang'andu HM, Fredriksen BN, Mutoloki S, Dalmo RA, Evensen Ø (2013) Antigen dose and humoral immune response correspond with protection for inactivated infectious pancreatic necrosis virus vaccines in Atlantic salmon (*Salmo salar* L). Vet Res 44(1):7
71. Ryan AA, Nambiar JK, Wozniak TM, Roediger B, Shklovskaya E, Britton WJ et al (2009) Antigen load governs the differential priming of CD8 T cells in response to the Bacille Calmette Guérin vaccine or *Mycobacterium tuberculosis* infection. J Immunol 182(11):7172
72. Henrickson SE, Mempel TR, Mazo IB, Liu B, Artyomov MN, Zheng H et al (2008) T cell sensing of antigen dose governs interactive behavior with dendritic cells and sets a threshold for T cell activation. Nat Immunol 9(3):282–291
73. Externest D, Meckelein B, Schmidt MA, Frey A (2000) Correlations between antibody immune responses at different mucosal effector sites are controlled by antigen type and dosage. Infect Immun 68(7):3830
74. Senkus E, Kyriakides S, Penault-Llorca F, Poortmans P, Thompson A, Zackrisson S et al (2013) Primary breast cancer: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. Ann Oncol 24(Suppl 6):vi7–vi23
75. Coates AS, Winer EP, Goldhirsch A, Gelber RD, Gnant M, Piccart-Gebhart M et al (2015) Tailoring therapies–improving the management of early breast cancer: St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2015. Ann Oncol 26(8):1533–1546
76. Teshome M, Hunt KK (2014) Neoadjuvant therapy in the treatment of breast cancer. Surg Oncol Clin N Am 23(3):505–523
77. Thompson AM, Moulder-Thompson SL (2012) Neoadjuvant treatment of breast cancer. Ann Oncol 23(Suppl 10):x231–x236
78. Park SY, Gonen M, Kim HJ, Michor F, Polyak K (2010) Cellular and genetic diversity in the progression of in situ human breast carcinomas to an invasive phenotype. J Clin Investig 120(2):636–644
79. Torres L, Ribeiro FR, Pandis N, Andersen JA, Heim S, Teixeira MR (2007) Intratumor genomic heterogeneity in breast cancer with clonal divergence between primary carcinomas and lymph node metastases. Breast Cancer Res Treat 102(2):143–155
80. Khan AM, Yuan Y (2016) Biopsy variability of lymphocytic infiltration in breast cancer subtypes and the ImmunoSkew score. Sci Rep 6:36231
81. Tay TKY, Thike AA, Pathmanathan N, Jara-Lazaro AR, Iqbal J, Sng ASH et al (2018) Using computer assisted image analysis to determine the optimal Ki67 threshold for predicting outcome of invasive breast cancer. Oncotarget 9(14):11619–11630
82. Yeong J, Lim JCT, Lee B, Li H, Ong CCH, Thike AA et al (2019) Prognostic value of CD8 + PD-1 + immune infiltrates and PDCD1 gene expression in triple negative breast cancer. J Immunother Cancer 7(1):34
83. Bin Seow DY, Lim J, Ong C, Lim J, Thike AA, Lim E et al (2019) Role of tissue resident memory CD8 + T cells in triple negative breast cancer. Pathology 51:S98
84. Bossard C, Jarry A, Colombeix C, Bach-Ngohou K, Moreau A, Loussouarn D et al (2006) Phosphohistone H3 labelling for histoprognostic grading of breast adenocarcinomas and computer-assisted determination of mitotic index. J Clin Pathol 59(7):706–710
85. Zbytek B, Cohen C, Wang J, Page A, Williams DJ, Adams AL (2013) Nottingham-defined mitotic score: comparison with visual and image cytometric phosphohistone H3 labeling indices and correlation with Oncotype DX recurrence score. Appl Immunohistochem Mol Morphol 21(1):48–53

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