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ORIGINAL ARTICLE

The role of Ki-67 in Asian triple negative breast cancers: a novel combinatory panel approach

An Sen Tan[1] · Joe Poe Sheng Yeong[2,3] · Chi Peng Timothy Lai[1] · Chong Hui Clara Ong[2] · Bernett Lee[3] · Jeffrey Chun Tatt Lim[2] · Aye Aye Thike[2] · Jabed Iqbal[2] · Rebecca Alexandra Dent[4] · Elaine Hsuen Lim[4] · Puay Hoon Tan[2]

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Abstract

The proliferation marker Ki-67 is frequently used to assess aggressiveness in the pathological evaluation of cancer, but its role remains uncertain in triple-negative breast cancer (TNBC). We aimed to quantify and localize Ki-67 expression in both epithelial and immune compartments in TNBC and investigate its association with clinicopathological parameters and survival outcomes. A total of 406 TNBC cases diagnosed between 2003 and 2015 at Singapore General Hospital were recruited. Using state-of-the-art, 7-colour multiplex immunofluorescence (mIF) tissue microarrays (TMAs) were stained to assess the abundance, density and spatial distribution of Ki-67-positive tumour cells and immune cells co-decorated with cytokeratin (CK) and leukocyte common antigen (CD45) respectively. Furthermore, MKI67 mRNA profiles were analysed using NanoString technology. In multivariate analysis adjusted for tumour size, histologic grade, age at diagnosis, and lymph node stage, a high Ki-67 labelling index (LI) > 0.3% was associated with improved disease-free survival (DFS; HR = 0.727; $p = 0.027$). High Ki-67-positive immune cell count per TMA was a favourable prognostic marker for both DFS (HR = 0.379; $p = 0.00153$) and overall survival (OS; HR = 0.473; $p = 0.0482$). The combination of high Ki-67 LI and high MKI67 expression was associated with improved DFS (HR = 0.239; $p = 0.00639$) and OS (HR = 0.213; $p = 0.034$). This study is among the first to highlight that Ki-67 is associated with favourable prognosis in an adjuvant setting in TNBC, and the mIF-based evaluation of Ki-67 expression on both tumour and immune cells represents a novel prognostic approach.

Keywords Breast cancer · Ki-67 · mRNA · Multiplex immunofluorescence

Background

Triple-negative breast cancer (TNBC) is defined by the absence of oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) protein expression and has an incidence of between 10 and 24% of all breast cancer diagnoses, varying with geographic and racial distributions [1]. In Singapore, TNBC accounts for 11% of all invasive breast cancer diagnoses [2]. TNBC has a disproportionately higher breast cancer-associated mortality due to its aggressive nature and the lack of targeted therapies [3–5]. However, identification of genomic subgroups with relatively favourable prognosis suggests that TNBC is a heterogeneous disease, with different biological behaviours [6]. Novel prognostic agents are therefore needed to stratify TNBCs, which may potentially advance the development of appropriate systemic measures for patients with more favourable prognoses.

The proliferation marker Ki-67 is a nuclear antigen expressed in all active phases of the cell cycle (G1, S, G2

An Sen Tan and Chi Peng Timothy Lai contributed equally to this work.

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✉ Puay Hoon Tan
tan.puay.hoon@singhealth.com.sg

Extended author information available on the last page of the article

and M-phase), but not in quiescent or resting cells [7]. Ki-67/MIB-1 monoclonal antibodies are reactive against Ki-67, with the percentage of immunoreactive tumour cell nuclei expressed as a labelling index (LI) [8–11]. Ki-67 is of prognostic value in multiple types of cancer; for example, a high Ki-67 LI is associated with poor clinical outcomes in prostate cancer [12] and gastrointestinal stromal tumours [13, 14]. However, a high Ki-67 LI is associated with favourable clinical outcomes in colorectal cancer (CRC) [15–17].

While a high Ki-67 LI is well-established as a marker of poor prognosis in breast cancer [18–20], the prognostic significance of Ki-67 in TNBC remains controversial. In 5 studies including 1201 TNBC patients, a high Ki-67 LI was associated with a poor prognosis [9, 21–24] (Supplementary Table 1), but none of these studies were performed in large cohorts comprising both early- and late-stage breast cancers or with modern treatments administered. Miyashita et al. (21) and Constantinou et al. [23] found that a high Ki-67 LI was associated with reduced disease-free survival (DFS) and overall survival (OS) in TNBC. However, these studies were limited in their statistical power due to small cohorts consisting of 102 and 84 cases respectively. Munzone et al. [9] only investigated node-negative cancers, and 315 of the 425 cases studied were treated with a CMF adjuvant chemotherapy regimen, which is less common in modern clinical practice. Wang et al. [21] found that overall, a high Ki-67 LI was associated with poor DFS and OS but that in the subpopulation of patients receiving carboplatin ($n = 58$), high Ki-67 LI was instead significantly associated with improved DFS. For Asian populations, while Pan et al.'s [24] study may be informative; it was based on a relatively small cohort of 156 patients. Findings from another 4 studies incorporating a total of 1125 patients found no association between a high Ki-67 LI and prognosis [25–28] (Supplementary Table 1). Notably, Hao et al. [27] concluded that high Ki-67 LI was associated with favourable prognosis in TNBC patients aged 50 or below.

There are several factors that may explain the discrepancies in these reports. For example, the use of different Ki-67 cut-offs [26] and high inter-observer variability in Ki-67 scoring [29] are likely to have an effect. At the 2017 St. Gallen International Expert Consensus Conference, the panel voiced concerns about the reproducibility of IHC when assessing Ki-67 expression and, thus, its use in clinical decisions [30]. It was suggested that calibration of a common Ki-67 scoring method to achieve high inter-laboratory reproducibility is pursued. Another issue is the dearth of knowledge regarding the prognostic significance of the immune cell proliferation in TNBC. Ki-67 is used as a proliferative marker in the field of immunology [31–33], and despite the importance of tumour infiltrating lymphocytes (TILs) in TNBC [31–35], no previous studies have investigated the significance of their proliferative activity in TNBC.

In light of the uncertain status of Ki-67 as a prognostic marker in TNBCs, the aim of our study was to assess the significance of Ki-67 proliferation in both carcinoma and TIL populations in an Asian TNBC cohort. The relationship between clinical outcomes, Ki-67 mRNA transcription levels, Ki-67 protein expression, CK (identifying carcinoma cells) and CD45 (identifying TILs) antigen expression was retrospectively analysed in an Asian cohort using multimodal technologies: conventional pathology, multiplex immunofluorescence (mIF), computer-assisted Vectra analysis and NanoString platforms. The use of computer-assisted Vectra analysis eliminated inter-observer variability and the use of mIF to evaluate CD45 and Ki-67 together allowed quantification of immune cell proliferation. We previously demonstrated that Ki-67 could be scored in a reproducible and quantitative manner with the digital Aperio ePathology image analysis system, negating inter-observer variability [36]. Thus, a secondary aim of the present study was to compare concordance of these results with those of our previous work.

Methods

Patients and tumours

A total of 406 TNBC patients diagnosed between 2003 and 2015 at the Department of Anatomical Pathology, Division of Pathology, Singapore General Hospital, were included in this study. 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. The tumour subtypes are summarized in Supplementary Table 2. Tumours were characterized according to the World Health Organization and American Society of Clinical Oncology-College of American Pathologists (ASCO-CAP) guidelines [37].

Median follow-up of the patients 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 occurred in 51 (12.6%) patients across the cohort. In total, 65 (16.0%) patients from the cohort succumbed to mortality.

TMA construction

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

Table 1 Cox proportional hazards analysis of MKI67 expression levels and survival outcomes in triple negative breast cancers, using data from the Cancer Genome Atlas and METABRIC, as well as the study cohort, and multivariate Cox proportional hazards analysis of survival outcomes using Ki-67 cell count in the epithelial compartment and immune compartment, as well as a combined MKI67 + Ki-67 index

Parameter	No. of events	No. of patients	HR (95% CI)	*p* value
The Cancer Genome Atlas dataset				
Overall survival				
Low MKI67 expression	9	21	Reference	
High MKI67 expression	6	68	0.156 (0.055, 0.442)	< 0.001[a]
METABRIC dataset				
Overall survival				
Low MKI67 expression	132	238	Reference	
High MKI67 expression	29	61	0.745 (0.497, 1.12)	0.155
Study cohort				
Disease-free survival				
Low MKI67 expression	59	200	Reference	
High MKI67 expression	13	58	0.470 (0.237, 0.930)	0.030[a]
Overall survival				
Low MKI67 expression	44	200	Reference	
High MKI67 expression	10	58	0.549 (0.251, 1.199)	0.132
Ki-67 cell count in epithelial compartment				
Disease-free survival				
≤ 2 cells	15	29	Reference	
> 2 cells	50	183	0.431 (0.228, 0.816)	0.010[a]
Overall survival				
≤ 2 cells	9	29	Reference	
> 2 cells	41	182	0.652 (0.291, 1.460)	0.298
Ki-67 cell count in immune compartment				
Disease-free survival				
≤ 2 cells	21	41	Reference	
> 2 cells	44	171	0.379 (0.208, 0.691)	0.002[a]
Overall survival				
≤ 2 cells	15	41	Reference	
> 2 cells	35	170	0.473 (0.225, 0.994)	0.048[a]
MKI67 + Ki-67 index with cut-off combination 1				
Disease-free survival				
Rest of cohort	49	150	Reference	
Both high	6	38	0.239 (0.086, 0.669)	0.006[a]
Overall survival				
Rest of cohort	38	150	Reference	
Both high	6	38	0.422 (0.164, 1.089)	0.075
MKI67 + Ki-67 index with cut-off combination 2				
Disease-free survival				
Rest of cohort	48	144	Reference	
Both high	7	44	0.227 (0.088, 0.588)	0.002[a]
Overall survival				
Rest of cohort	38	144	Reference	
Both high	6	44	0.314 (0.119, 0.828)	0.019[a]

MKI67 + Ki-67 index cut-off combination 1: Ki-67 cut-off, 0.3% and MKI67 cut-off, 2.335557131

MKI67 + Ki-67 index cut-off combination 2: Ki-67 cut-off, 0.23% and MKI67 cut-off, 2.3085

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

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 [38–48]. FFPE tissue sections were processed according to a standard immunohistochemistry protocol, as previously described [49, 50]. Slides were labelled with primary antibodies against Ki-67, CK and CD45, followed by appropriate secondary antibodies (as presented in Supplementary Table 3), prior to application of a fluorophore-conjugated tyramide signal amplification buffer (PerkinElmer, Inc.). DAPI was used as a nuclear counterstain, and images were acquired using a Vectra 3 pathology imaging system microscope (PerkinElmer, Inc.) and analysed using inForm software (version 2.4.1; PerkinElmer, Inc.) [39, 51, 52].

RNA extraction and NanoString measurement of *PDCD1* and *CD274* expression

RNA was extracted from unlabelled FFPE sections (10 μm thick) using an RNeasy FFPE kit according to the manufacturer's protocol (Qiagen GmbH, Hilden, Germany) on a QIAcube automated sample preparation system (Qiagen GmbH) and was quantified using an Agilent 2100 Bioanalyzer system (Agilent Technologies, Santa Clara, CA, USA). Functional RNA (100 ng; > 300 nucleotides) was assayed on the nCounter MAX Analysis System (NanoString Technologies, Inc., Seattle, WA, USA). The NanoString counts were normalized using positive control probes and housekeeping genes, as previously reported [50]. The count data were then logarithmically transformed as previously described prior to further analysis [49]. $p < 0.05$ was considered to indicate a statistically significant difference.

Validation, follow-up and statistical analysis

Follow-up data were obtained from medical records. DFS and OS were defined as the time from diagnosis to recurrence or death/date of last follow-up, respectively. There was no follow-up data for 108 (26.6%) patients, and these patients were excluded from the survival analysis. Statistical analysis was performed using RStudio 1.1.456 running R 3.5.0 (R-core Team, R Foundation for Statistical Computing, Vienna, Austria) [53, 54]. Data import and processing were supported by the packages openxlsx, ggplot2, dplyr, tidyr, stringr and survminer [54–61]. 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 Ki-67 expression and the NanoString MKI67 counts on survival, after adjusting for clinicopathological parameters that included patient age at diagnosis, tumour size, tumour grade and lymph node status. Gene expression and survival data for METABRIC, EGAS00001001753 from the European Genome–phenome Archive [62] and The Cancer Genome Atlas [63] were obtained from cBioPortal [64] for validation purposes, after filtering for TNBC samples.

Maximally selected rank statistics [65] were applied using the maxstat R package to find optimal cut-off points with good prediction of survival outcomes for continuous variables. When analysing a combination of two variables (*MKI67* gene expression and Ki-67 LI), each variable was analysed independently by applying a cut-off point to identify TMAs with either high or low expression and subsequently organized into 4 different groups: those with high expression of both variables, 2 groups with high expression of either variable only and a group with low expression of both variables. Finally, Kaplan–Meier survival analysis and multivariate Cox regression were performed on this processed data as outlined above. A computational algorithm was devised to iteratively apply all possible combinations of the two cut-off points, with multivariate Cox proportional hazards regression performed at each iteration of the algorithm, and the resulting p value recorded. The combination of cut-off points that produced the lowest p value was then selected. A summary of all the ranges, means, medians and cut-offs used for all the variables analysed in this study is presented in Supplementary Table 4. Models were compared using the increment in log-likelihood of the models ($\Delta LR\chi^2$) using a likelihood ratio test. $p < 0.05$ was considered to indicate a statistically significant difference.

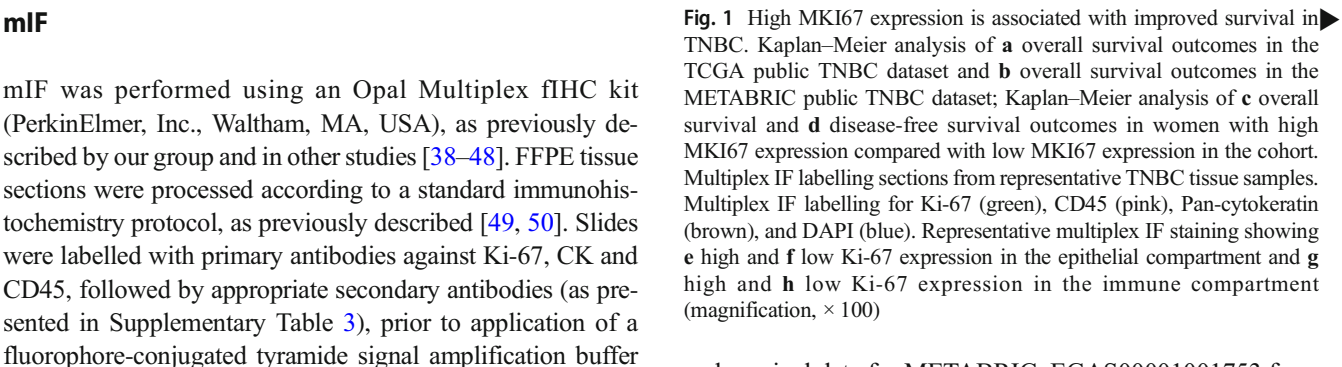
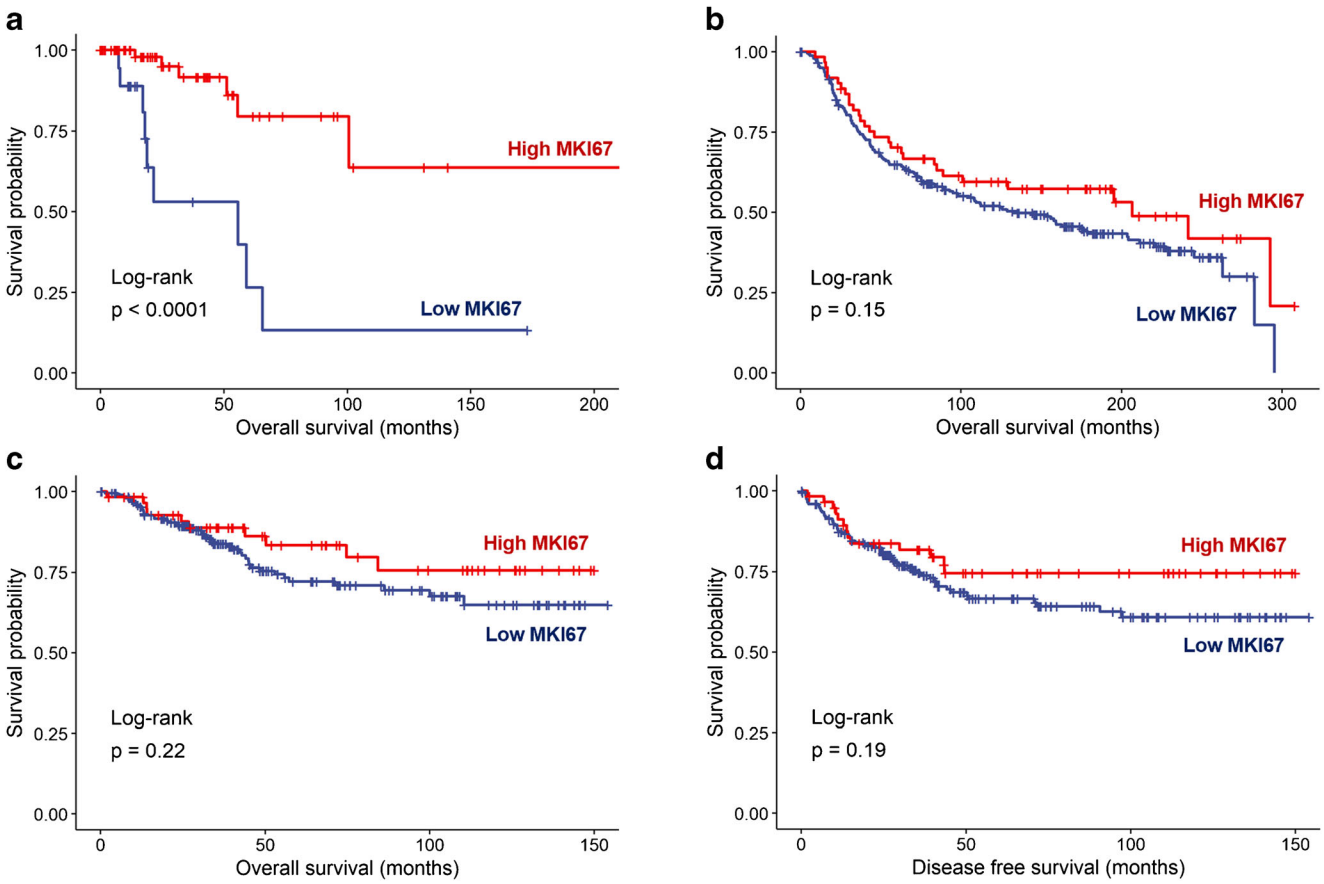


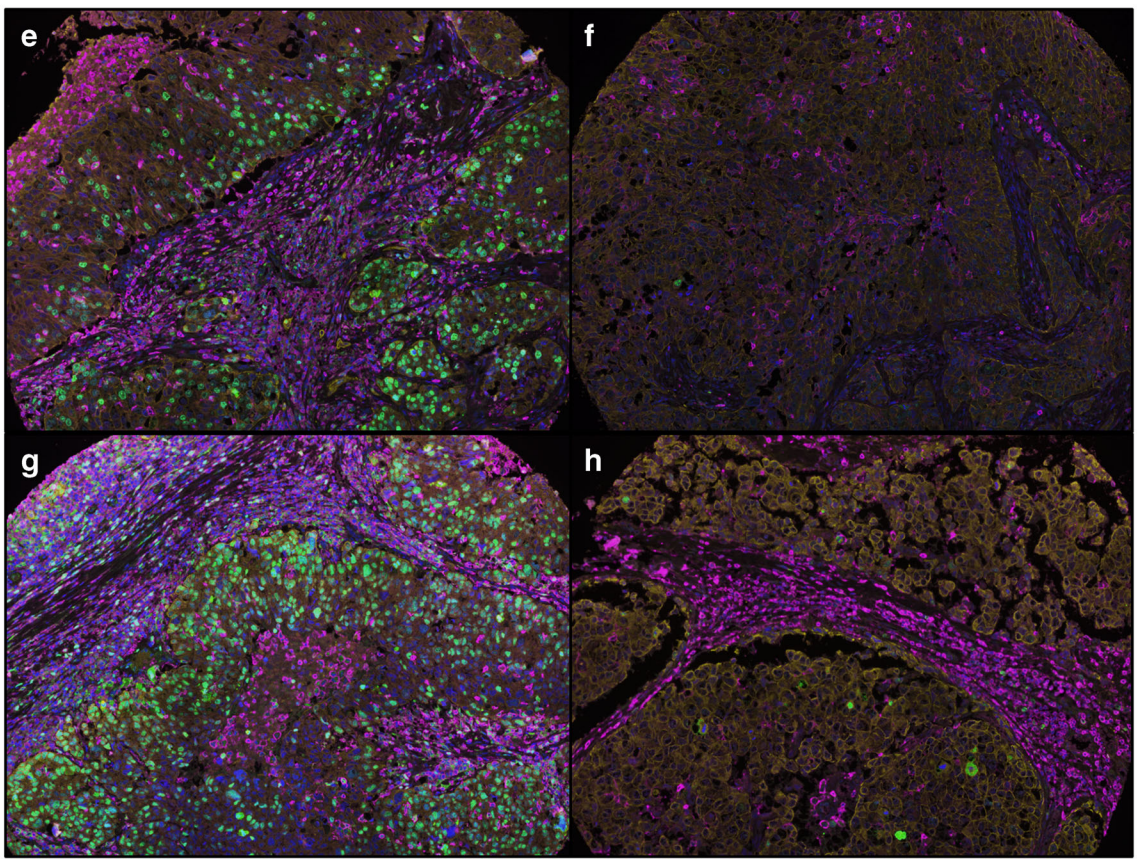
Fig. 1 High MKI67 expression is associated with improved survival in TNBC. Kaplan–Meier analysis of **a** overall survival outcomes in the TCGA public TNBC dataset and **b** overall survival outcomes in the METABRIC public TNBC dataset; Kaplan–Meier analysis of **c** overall survival and **d** disease-free survival outcomes in women with high MKI67 expression compared with low MKI67 expression in the cohort. Multiplex IF labelling sections from representative TNBC tissue samples. Multiplex IF labelling for Ki-67 (green), CD45 (pink), Pan-cytokeratin (brown), and DAPI (blue). Representative multiplex IF staining showing **e** high and **f** low Ki-67 expression in the epithelial compartment and **g** high and **h** low Ki-67 expression in the immune compartment (magnification, × 100)

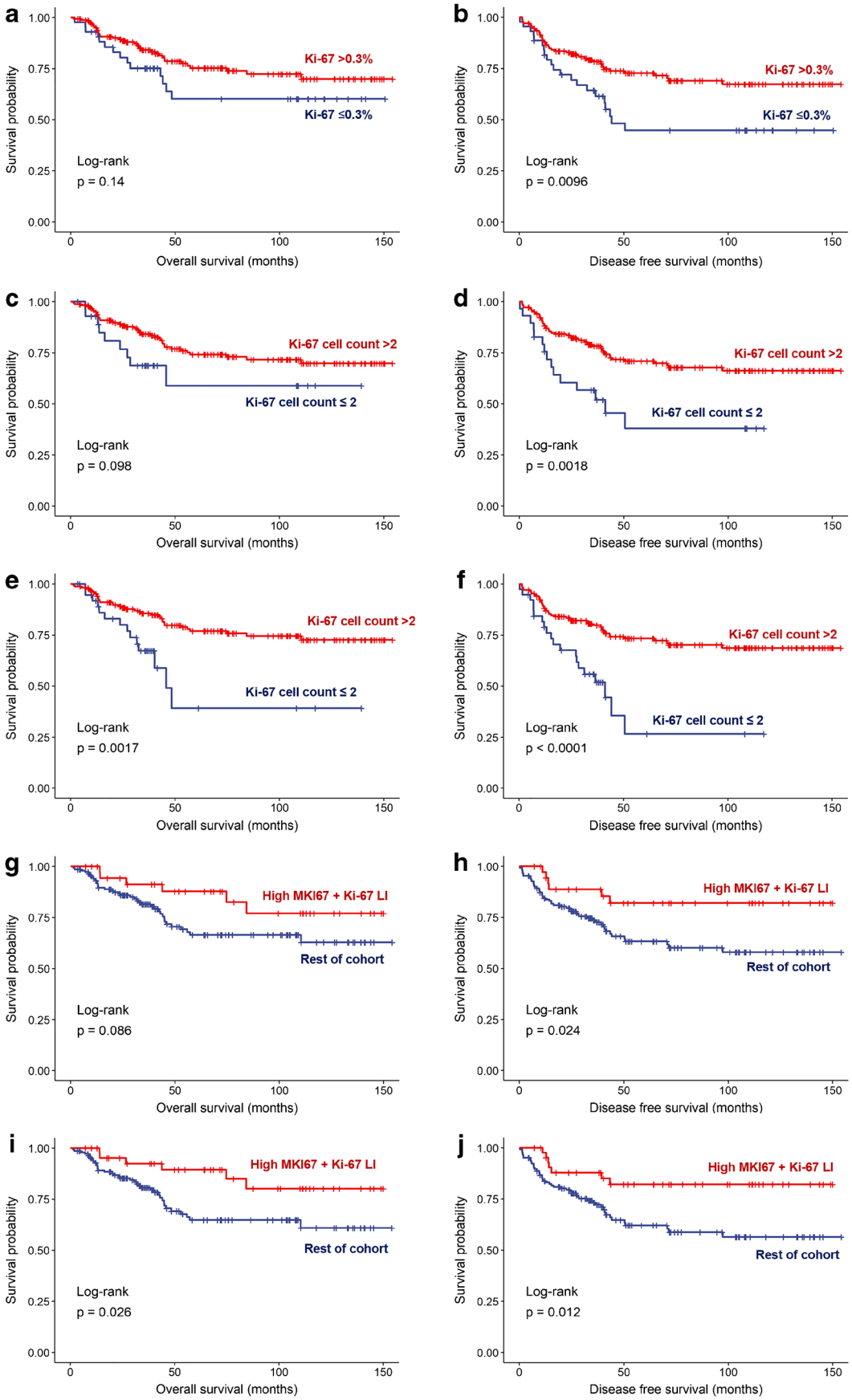
Results

MKI67 gene expression is associated with improved overall survival in public datasets

Initial univariate analysis of a public dataset from The Cancer Genome Atlas, comprising 89 TNBC cases, revealed a significant positive association between increased *MKI67* expression and favourable OS (HR = 0.156; 95% CI 0.0551–0.442; $p = 0.000470$; Table 1). Kaplan–Meier analysis also revealed a significant difference in OS (log-rank test, $p < 0.0001$; Fig. 1a). However, univariate analysis of *MKI67* expression data from the publicly available METABRIC dataset failed to


a
Survival probability
1.00
0.75
0.50
0.25
0.00
High MKI67
Low MKI67
Log-rank
p < 0.0001
0
50
100
150
200
Overall survival (months)
b
Survival probability
High MKI67
Low MKI67
Log-rank
p = 0.15
0
100
200
300
Overall survival (months)
c
Survival probability
High MKI67
Low MKI67
Log-rank
p = 0.22
0
50
100
150
Overall survival (months)
d
Survival probability
High MKI67
Low MKI67
Log-rank
p = 0.19
0
50
100
150
Disease free survival (months)


e
f
g
h


a
Survival probability
1.00
0.75
0.50
0.25
0.00
Ki-67 >0.3%
Ki-67 ≤0.3%
Log-rank
p = 0.14
0
50
100
150
Overall survival (months)
b
Survival probability
Ki-67 >0.3%
Ki-67 ≤0.3%
Log-rank
p = 0.0096
Disease free survival (months)
c
Survival probability
Ki-67 cell count >2
Ki-67 cell count ≤ 2
Log-rank
p = 0.098
Overall survival (months)
d
Survival probability
Ki-67 cell count >2
Ki-67 cell count ≤ 2
Log-rank
p = 0.0018
Disease free survival (months)
e
Survival probability
Ki-67 cell count >2
Ki-67 cell count ≤ 2
Log-rank
p = 0.0017
Overall survival (months)
f
Survival probability
Ki-67 cell count >2
Ki-67 cell count ≤ 2
Log-rank
p < 0.0001
Disease free survival (months)
g
Survival probability
High MKI67 + Ki-67 LI
Rest of cohort
Log-rank
p = 0.086
Overall survival (months)
h
Survival probability
High MKI67 + Ki-67 LI
Rest of cohort
Log-rank
p = 0.024
Disease free survival (months)
i
Survival probability
High MKI67 + Ki-67 LI
Rest of cohort
Log-rank
p = 0.026
Overall survival (months)
j
Survival probability
High MKI67 + Ki-67 LI
Rest of cohort
Log-rank
p = 0.012
Disease free survival (months)

◀ **Fig. 2** High Ki-67 expression is associated with improved survival in TNBC. Kaplan–Meier analysis of **a** overall survival and **b** disease-free survival outcomes in women with high versus low Ki-67 labelling index, Kaplan–Meier analysis of **c** overall survival and **d** disease-free survival outcomes in women with high versus low Ki-67 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 versus low Ki-67 cell count per high-power field in the immune compartment. A combination of high MKI67 expression and high Ki-67 labelling index (LI) is associated with improved survival in TNBC. Kaplan–Meier analysis of **g** overall survival and **h** disease-free survival outcomes in women with concurrently high MKI67 expression and Ki-67 labelling index versus the rest of the cohort using a Ki-67 LI cut-off of 0.3%; Kaplan–Meier analysis of **i** overall survival and **j** disease-free survival outcomes in women with concurrently high MKI67 expression and Ki-67 labelling index versus the rest of the cohort using a Ki-67 LI cut-off of 0.23% and MKI67 cut-off 2.3085

reveal an association between increased *MKI67* expression and OS in 299 cases of TNBC (HR = 0.745; 95% CI 0.497–1.12, $p = 0.155$; Table 1). Kaplan–Meier survival analysis also found no significant difference (Fig. 1b).

MKI67 gene expression is associated with favourable disease-free survival in an Asian TNBC cohort

Kaplan–Meier survival analysis revealed that neither DFS nor OS significantly differed between the high and low *MKI67* expression groups (Fig. 1c and d). However, multivariate analysis adjusted for tumour size, histologic grade, age at diagnosis and lymph node status revealed that high *MKI67* expression > 2.335557131 was significantly associated with favourable DFS (HR = 0.470; 95% CI 0.237–0.930, $p = 0.030$; Table 1). However, *MKI67* expression was not significantly associated with OS (HR = 0.549; 95% CI 0.251–1.199, $p = 0.132$; Table 1).

Table 2 Comparison of the Ki-67 index in the epithelial compartment between the PerkinElmer Vectra system and the Aperio and Definiens systems

Parameter	PerkinElmer Vectra vs. Definiens Tissue Studio	PerkinElmer Vectra vs. Aperio ePathology IHC Nuclear Image Analysis
Pearson's *r* correlation coefficient	0.4974	0.488
95% CI	0.2157–0.7026	0.204–0.697
p value (two-tailed) for significance of correlation	0.0013[a]	0.002[a]

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

mIF Vectra image analysis results are concordant with previously validated image analysis platforms

We adopted an automated quantitative multiplex IF (mIF) platform that we and others have previously reported [38–49]. Opal mIF staining for Ki-67, CK and CD45 was performed on TNBC tumour sections, followed by image acquisition with a Vectra 3 pathology imaging system and image analysis with inForm software (Fig. 1e–h). The Ki-67 scoring results generated by the PerkinElmer Vectra image analysis platform were compared with those from the Definiens Tissue Studio platform and the Aperio ePathology Immunohistochemistry Nuclear Image Analysis platform, work that we previously published [36]. There was good concordance between the results, with Pearson's *r* correlation coefficient values of 0.4974 (95% CI 0.2157–0.7026, $p = 0.0013$) and 0.4884 (95% CI 0.2044–0.6966, $p = 0.0016$), respectively (Table 2).

Ki-67 labelling index is associated with improved disease-free survival

Kaplan–Meier survival analysis revealed that a Ki-67 LI > 0.3% was associated with significantly improved disease-free survival (log-rank $p = 0.0096$), but Ki-67 LI was not significantly associated with OS (Fig. 2a and b). Multivariate analysis revealed that Ki-67 LI was an independent favourable prognostic factor for DFS (HR = 0.727; 95% CI 0.549–0.964, $p = 0.027$). Positive axillary lymph node (ALN) status was also associated with shorter DFS, but other clinicopathological parameters such as tumour size and histological grade did not influence prognosis (Table 3). However, Ki-67 LI was not significantly associated with OS (HR = 0.919; 95% CI 0.648–1.303, $p = 0.635$); only increased ALN status was associated with shorter OS (Table 3).

Additional Kaplan–Meier analysis was then performed, applying Ki-67 LI cut-offs ranging from 10 to 40%, as previously reported in other studies concerning TNBC (Supplementary Figs. 1 and 2). However, Ki-67 LI was not associated with DFS or OS when using these additional cut-offs. The fact that the 0.3% cut-off resulted in significance when previous cut-offs failed to do so may be because the naked eye is unable to accurately score precise values; hence, an automated platform such as the QmIF would be superior in this regard.

Total Ki-67 cell count per high power field is associated with improved survival in TNBC

A fine cut-off of 0.3% may not be practical in routine clinical practice. For this reason, we also investigated cell count per high-power field, which may be more conducive for routine application. Multivariate analysis of DFS with additional cut-offs of 5, 10 and 15 cells is presented in the supplementary

Table 3 Multivariate Cox regression for overall survival and disease-free survival using Ki-67 protein expression and clinicopathological parameters

Clinicopathological parameters	Overall survival		Disease-free survival	
	HR (95% CI)	*p* value	HR (95% CI)	*p* value
Tumour size				
T1	Reference		Reference	
T2	1.490 (0.631–3.519)	0.363	1.230 (0.619–2.444)	0.555
T3–4	2.336 (0.835–6.534)	0.106	2.222 (0.941–5.238)	0.069
ALN status				
N0	Reference		Reference	
N1	2.740 (1.180–6.360)	0.019[a]	2.629 (1.353–5.109)	0.004[a]
N2	5.008 (2.204–11.377)	0.000[a]	2.447 (1.126–5.316)	0.024[a]
N3	18.853 (6.436–55.228)	0.000[a]	9.645 (3.711–25.070)	<0.001[a]
Grade				
I	Reference		Reference	
II	0.276 (0.0418–1.828)	0.182	1.176 (0.129–10.689)	0.885
III	0.573 (0.121–2.705)	0.481	1.275 (0.163–9.992)	0.817
Age at diagnosis				
Age (years)	0.998 (0.970–1.028)	0.911	0.993 (0.967–1.019)	0.579
Ki-67 (with cut-off = 0.3%)				
Low	Reference		Reference	
High	0.919 (0.648, 1.303)	0.635	0.727 (0.549, 0.964)	0.027[a]

ALN axillary lymph node status

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

section (Supplementary Table 5). Cut-offs using 2, 5, 10 or 15 cells were not significantly associated with OS and are not presented.

Kaplan–Meier survival analysis (Supplementary Fig. 3) showed that a total Ki-67 cell count of > 3 cells per high-power field, regardless of localization, was associated with significantly increased DFS (log-rank $p = 0.0017$) and OS (log-rank $p = 0.02$). Multivariate analysis adjusted for tumour size, histological grade, age at diagnosis and axillary lymph node status revealed that a total Ki-67 cell count of > 3 cells per high-power field was an independent favourable prognostic marker for DFS (HR = 0.508; 95% CI 0.258–0.999, $p = 0.0497$; Supplementary Table 6) but was not significantly associated with OS (HR = 0.587; 95% CI 0.262–1.312, $p = 0.194$; Supplementary Table 6).

Ki-67 cell count in both the epithelial and immune compartments is an independent favourable prognostic marker in TNBC

Next, the influence of Ki-67 localization was investigated. Kaplan–Meier survival analysis revealed that a Ki-67 cell count of > 2 cells per high-power field (1000 μm × 750 μm) within the malignant epithelial compartment was associated with significantly increased DFS (log-rank $p < 0.0018$) but was not associated with OS (log-rank $p = 0.098$; Fig. 2c and d). Furthermore, Kaplan–Meier survival analysis showed that a Ki-67 cell count of > 2 cells per high-power field (1000 μm × 750 μm) within the immune compartment was associated with significantly increased DFS (log-rank $p < 0.0001$) and OS (log-rank $p = 0.0017$; Fig. 2e and f). Confirming these results, multivariate analysis adjusted for tumour size, histological grade, age at diagnosis and axillary lymph node status revealed that a Ki-67 positive cell count of > 2 cells per high-power field in the malignant epithelial compartment was an independent favourable prognostic marker for DFS (HR = 0.431; 95% CI 0.228–0.816, $p = 0.00977$; Table 1) but not OS (HR = 0.612; 95% CI 0.291–1.460, $p = 0.298$; Table 1). For the immune compartment, a Ki-67 positive cell count of > 2 cells per high-power field was revealed to be an independent favourable prognostic marker for both DFS (HR = 0.379; 95% CI 0.208–0.691, $p = 0.00153$; Table 1) and OS (HR = 0.473; 95% CI 0.225–0.994, $p = 0.0482$; Table 1).

MKI67 and Ki-67 LI form an independent favourable prognostic panel offering enhanced prognostic value

We compared patients who concurrently exhibited a high Ki-67 LI and high *MKI67* expression with all other patients in the cohort. Cut-offs of 0.3% and 2.335557131 for Ki-67 LI and *MKI67* expression, respectively, were applied for the analysis. Kaplan–Meier survival analysis (Fig. 2g and h) showed that a combination of high Ki-67 LI and high *MKI67* expression was significantly associated with improved DFS (log-rank $p =$

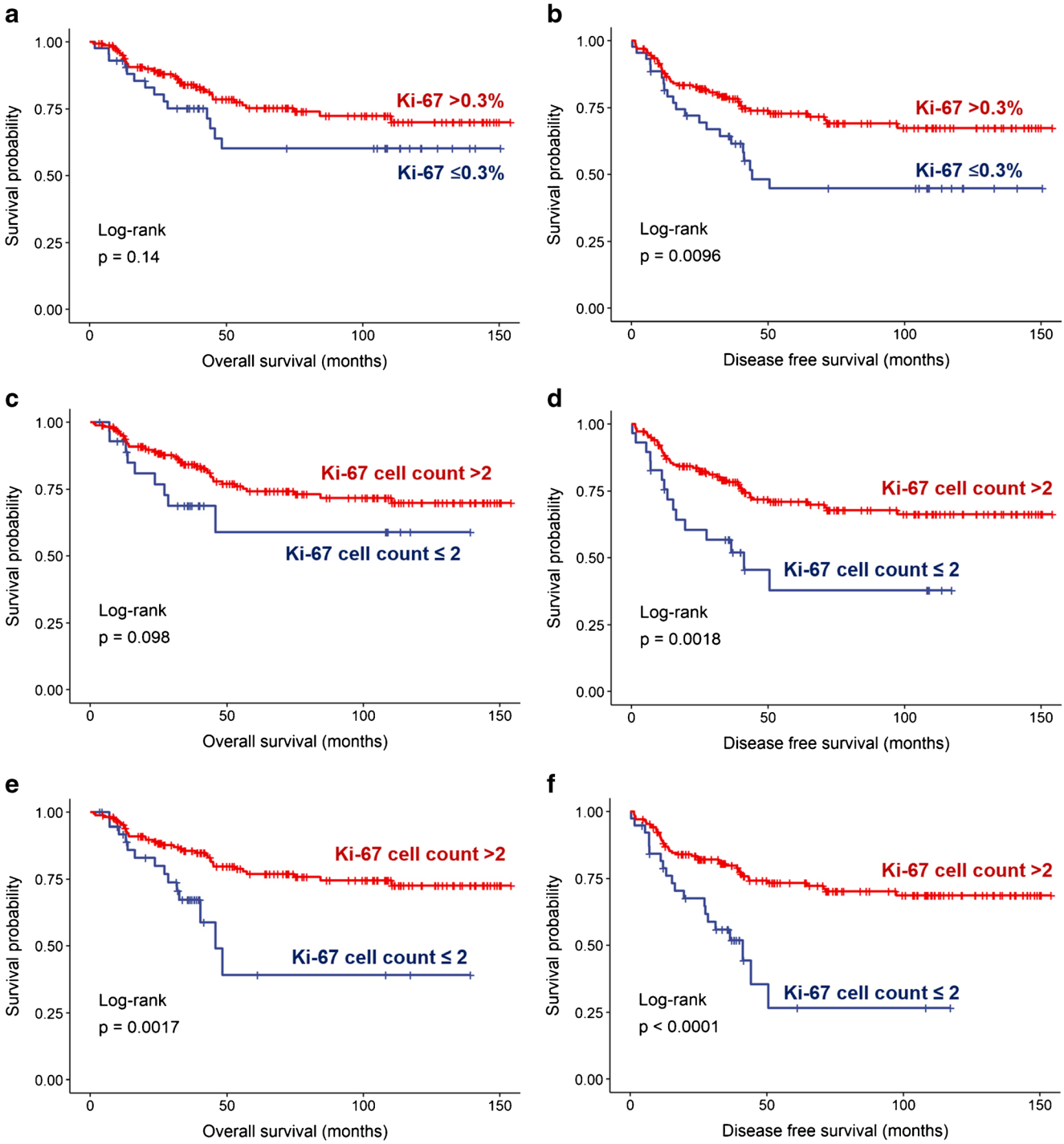


Fig. 3 Survival correlation

0.024), but there was no significant association with OS (log-rank $p = 0.086$). Multivariate analysis adjusted for tumour size, histological grade, age at diagnosis and axillary lymph node status revealed that a combination of a high Ki-67 LI (> 0.3%) and high MKI67 expression represented an independent favourable prognostic marker for DFS (HR = 0.239; 95% CI 0.0857–0.669, $p = 0.00639$; Table 1) but was not significantly associated with OS (HR = 0.422; 95% CI 0.164–1.089, $p = 0.0746$; Table 1).

For this reason, additional analysis using a Ki-67 LI cut-off of 0.23% and an *MK167* cut-off of 2.3085 was performed; both cut-off points were computationally derived as optimal using an iterative algorithm as described in 'Methods'. With these cut-offs applied, Kaplan–Meier analysis (Fig. 2i and j) revealed that a combination of high Ki-67 LI and high MKI67

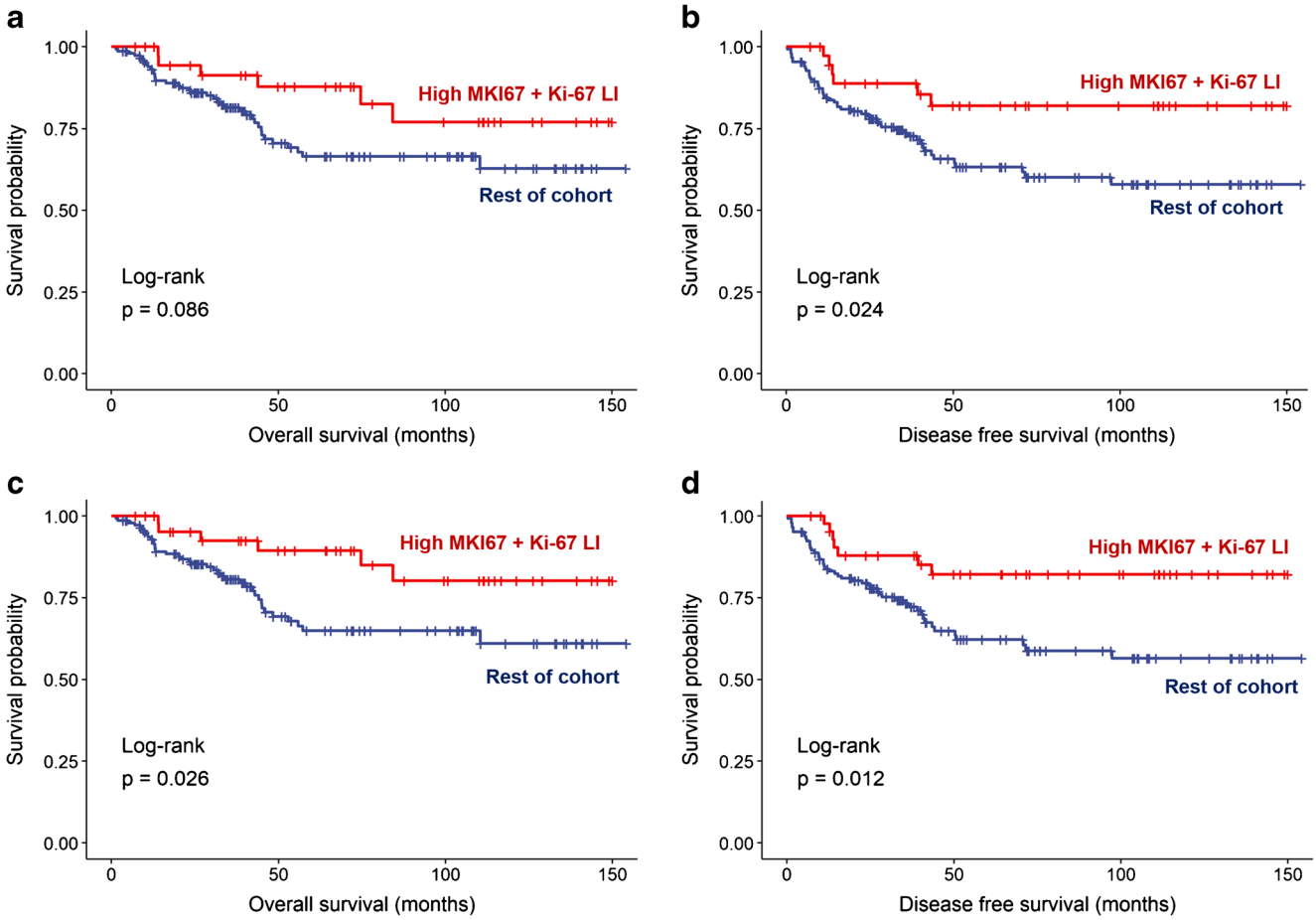


Fig. 4 Survival probability

expression was significantly associated with improved DFS (log-rank $p = 0.019$) and OS (log-rank $p = 0.022$). Multivariate analysis demonstrated that the combination of Ki-67 LI > 1.2% and high *MKI67* expression was an independent favourable prognostic marker for both DFS (HR = 0.162; 95% CI 0.039–0.672, $p = 0.012$; Table 1) and OS (HR = 0.213; 95% CI 0.051–0.889, $p = 0.034$; Table 1), after adjustment for tumour size, histological grade, age at diagnosis and axillary lymph node status. The combination of *MKI67* and Ki-67 LI offers enhanced prognostic value, exceeding that of any single clinicopathological parameter or any individual marker presented in this paper.

Discussion

The major conclusion of our study was that elevated Ki-67 expression is associated with favourable prognosis in TNBC. This result was corroborated through multiple layers of analysis, including RNA analysis of *MKI67* expression and mIF quantification of Ki-67 LI. The combination of Ki-67 LI with *MKI67* expression represented an independent predictor of favourable prognosis. The large patient cohort allowed us to demonstrate that the prognostic significance of Ki-67 in TNBC was independent of age, tumour size, tumour grade and axillary lymph node status. To our best knowledge, this study is among the first to highlight that Ki-67 is associated with favourable prognosis in women with TNBC, independent of age.

For the first level of analysis, molecular data was used to determine the prognostic significance of Ki-67. First, we analysed publicly available data, namely the METABRIC and The Cancer Genome Atlas (TCGA) databases. While *MKI67* expression was associated with favourable prognosis in TNBC in the METABRIC database, it was found to have no association with prognosis in the TCGA database. Analysis of our own cohort revealed that *MKI67* was an independent marker of favourable prognosis in TNBC, aligning with results obtained from the METABRIC database. Although these results are at the transcriptional level, they contradict prevailing understanding that high Ki-67 LI is associated with poor prognosis [9, 21–24]. The association of high Ki-67 LI with favourable prognosis in TNBC was further confirmed by our second layer of analysis, where mIF was used to quantify Ki-67 LI.

To further validate these conclusions, we added a third layer of analysis: the combination of mIF with genetic analysis in the form of *MKI67* gene expression data. Combinations

of protein and genetic analysis are occasionally used for diagnostic purposes: for example, several ASCO/CAP guidelines recommend that breast carcinomas may require both immunohistochemistry and in situ cytogenetic analysis to determine HER2 status [66]. Many advocate screening using immunohistochemistry, and with indeterminate results, to be confirmed with FISH testing [67–73]. When Duchrow et al. [74] studied Ki-67 in CRC, they found that tumours with a high Ki-67 LI but low *MKI67* mRNA expression proliferated at a slower rate than expected. They suggested that a significant fraction of non-cycling tumour cells expressed Ki-67 in at least a third of CRC cases, resulting in slower proliferation than that predicted by Ki-67 expression alone. Therefore, we combined mIF with *MKI67* gene expression analysis to exclusively quantify those cells that were truly proliferating. When Ki-67 LI was combined with *MKI67* expression, we found that a combinational high Ki-67 LI and high *MKI67* expression was independently significantly associated with improved prognosis, substantiating our previous results.

Our results contrast with some previous studies investigating the prognostic significance of *MKI67* and Ki-67 expression in breast cancers as a whole. Bertucci et al [75] found that high *MKI67* expression was associated with poor prognosis in a cohort of all subtypes of node-positive breast cancers, while other authors discovered that although breast cancers with high Ki-67 expression were more sensitive to chemotherapy [76–80], this was also paradoxically associated with poor prognosis [81, 82]. These differences may be due to the unique characteristics of TNBC when compared to other types of breast cancer. While TNBC is relatively aggressive [83–87] with high proliferation rates [78, 83], they are also more sensitive to chemotherapy than other types of breast cancer [88–90]. Thus, TNBCs with high Ki-67 expression may be more chemosensitive, explaining their relatively better prognosis. This phenomenon of highly proliferative tumours being more responsive to chemotherapy and thus having improved prognosis has been previously reported in CRC, where high Ki-67 LI predicted favourable prognosis [16, 91].

Our results differ with previous work investigating the prognostic significance of Ki-67 expression in TNBCs (Supplementary Table 1). We propose several reasons for this. One important reason might be heterogeneity across the study cohorts, particularly relevant in the context of TNBCs. It is known that heterogeneity of TNBCs can contribute to poor response to chemotherapy [6]. Furthermore, ethnic differences may play a role in tumour behaviour and prognosis. For instance, in a study comparing non-Asian and Taiwanese TNBC patients, Tseng et al. found significant differences in the proportion of molecular subtypes of TNBC [92]. Accordingly, differences in patient cohorts, such as studies performed in Asian as compared to Caucasian populations, may partly explain these discrepancies.

Notably, our study demonstrates the prognostic value of Ki-67 in the immune compartment of TNBC, with high Ki-67 expression among immune infiltrates auguring an improved prognosis. Studies have highlighted the prognostic impact of the immune microenvironment in TNBC, which has greater abundance of TILs compared with other breast cancer subtypes [31–35]. In breast cancers in general, a high frequency of TILs is associated with reduced survival in patients with ER^+ breast tumours, while in TNBCs, the same is linked to significantly improved survival [85, 93]. Previous studies may have been confounded by the quantification of Ki-67 index on both immune and tumour cells without the ability to discriminate the two cell types accurately. This may partly account for the contradictory conclusions concerning the prognostic significance of Ki-67 in breast cancers.

A key limitation surrounding the value of Ki-67 assessment is the high inter-observer variability in immunohistochemical interpretation [29, 94]. There is currently no universally agreed cut-off value that distinguishes a high Ki-67 LI from a low Ki-67 LI. Some propose a value of 10% [95], while others favour a threshold of 30% [96]. This difference between studies can be attributed to inter-observer variability in visual assessment methods, such as 'eye-ball' visual estimation of Ki-67 positivity or quantitative cell-counting methods [29, 97]. Computer-assisted image analysis has been shown to ameliorate this problem, allowing for improved reproducibility of Ki-67 LI across studies [36, 98]. This should aid the selection of more universal cut-off values. In the present study, the PerkinElmer Vectra image analysis platform was used to score Ki-67 LI. When compared to other image analysis platforms, namely the Definiens Tissue Studio and the Aperio ePathology Immunohistochemistry Nuclear Image Analysis platform, we found that the results had a moderately strong agreement. This suggests that results from quantitative mIF may alleviate inter-observer variability that currently plagues manual Ki-67 scoring. An additional advantage of mIF is the ability to delineate proliferation in both tumour and immune cells, with our results indicating that both predicted improved survival. This may be because proliferating immune cells actively attack and kill tumour cells, while proliferating tumour cells are more vulnerable to chemotherapy.

Our findings may help individualize the application of adjuvant chemotherapy. Patients with TNBC with low Ki-67 expression may benefit more from treatment with alternative drug regimens rather than those targeting rapidly proliferating cells. Although our study investigated TNBC treated with adjuvant chemotherapy, the conclusions may potentially be applicable to TNBCs treated using a neoadjuvant approach. Neoadjuvant therapy is well-established for selected high-risk breast cancers, including larger tumours and locally advanced disease initially ineligible for resection [99, 100]. In a landmark German study by Fasching et al [101], Ki-67 was found to have both predictive and prognostic value in a cohort of 552

patients receiving neoadjuvant treatment for all molecular subtypes of invasive breast cancer. High Ki-67 expression was associated with significantly improved pathological complete response, as well as with favourable DFS and OS. This finding parallels our conclusions. Further studies in the neoadjuvant setting of TNBCs, including in Asian populations, will be helpful.

Another potential application of this study is in the use of Ki-67 in immunotherapy. Proliferation of immune cells, as measured by immune cell Ki-67 expression, was revealed to be of prognostic significance, lending credence to the use of Ki-67 scoring of immune cells in TNBC. Future work could include increased characterization of proliferating immune cells to further elucidate tumour-host immune reactions and guide future immunotherapy approaches in TNBC.

Limitations

One limitation of our study is the use of TMA cores, with possible non-representative sampling of the potentially heterogeneous tumours. While we concede that it would be ideal to have analysis of whole sections in addition to our analysis of TMA cores, there is evidence suggesting high concordance between TMA cores and whole sections, which supports the utility of TMAs for initial discovery. For instance, Kobierzycki et al. reported excellent concordance between TMAs and standard sections ($r = 0.91$) for Ki67 expression in a study investigating 51 cases of invasive breast cancer [102]. Excellent agreement between TMA cores and whole slides was also found by Ruiz et al. [103], Muftah et al. [104] and Batistatou et al. [105]. Furthermore, in a study investigating routine biomarker assessment in breast cancer, Thomson et al. [106] found no statistically significant decrease in concordance between TMAs and whole section results with the use of 2 cores instead of 4. Our group (Tay et al. [36]) reported that using TMA cores to determine the optimal threshold for Ki-67 yielded survival correlations and thresholds that mirrored those previously reported in the literature based on whole slides (Figs. 3 and 4).

Presently, routine histopathology practice does not involve the evaluation of TMAs. We concede that a Ki-67 LI cut-point of 0.3% is challenging to apply clinically unless quantitative scoring of Ki-67 is performed. In consideration of this constraint, we presented an alternative measure of the Ki-67 index comprising the number of Ki-67 positive cells per high power field which would be more conducive for clinical application. It is difficult to directly extrapolate our cut-points to clinical practice at this stage, and further, validation is warranted prior to translation to routine clinical practice where whole tissue sections from either core biopsies or excision specimens are evaluated.

Conclusions

In conclusion, our study established that high Ki-67 expression is associated with improved prognosis in TNBC through multiple levels of analysis including RNA analysis of *MKI67* expression and mIF quantification of the Ki-67 LI. Given that Ki-67 can be reliably scored using computer-assisted image analysis, a prognostic system including this marker may aid treatment selection for patients with TNBCs.

Authors' 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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Data availability The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standards

Ethics approval and consent to participate 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).

Competing interests The authors declare that they have no competing interests.

Abbreviations ALN, axillary lymph node; CD45, leukocyte common antigen; CK, cytokeratin; CRC, colorectal cancer; DFS, disease-free survival; ER, oestrogen receptor; HER2, human epidermal growth factor receptor 2; LI, labelling index; mIF, multiplex immunofluorescence; OS, overall survival; PR, progesterone receptor; TIL, tumour infiltrating lymphocytes; TMA, tissue microarray; TNBC, triple negative breast cancer

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Affiliations

An Sen Tan[1] · Joe Poe Sheng Yeong[2,3] · Chi Peng Timothy Lai[1] · Chong Hui Clara Ong[2] · Bernett Lee[3] · Jeffrey Chun Tatt Lim[2] · Aye Aye Thike[2] · Jabed Iqbal[2] · Rebecca Alexandra Dent[4] · Elaine Hsuen Lim[4] · Puay Hoon Tan[2]

An Sen Tan
TANA0031@e.ntu.edu.sg

Joe Poe Sheng Yeong
joe.yeong.p.s@sgh.com.sg

Chi Peng Timothy Lai
CLAI003@e.ntu.edu.sg

Chong Hui Clara Ong
clara.ong.c.h@sgh.com.sg

Bernett Lee
bernett_lee@immunol.a-star.edu.sg

Jeffrey Chun Tatt Lim
jeffrey.lim.c.t@sgh.com.sg

Aye Aye Thike
daw.aye.aye.thike@sgh.com.sg

Jabed Iqbal
jabed.iqbal@singhealth.com.sg

Rebecca Alexandra Dent
rebecca.dent@duke-nus.edu.sg

Elaine Hsuen Lim
elaine.lim.hsuen@singhealth.com.sg

1 Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore, Singapore

2 Division of Pathology, Singapore General Hospital, 20 College Road, Academia, Level 7, Diagnostics Tower, Singapore 169856, Singapore

3 Singapore Immunology Network (SIgN), Agency of Science, Technology and Research (A*STAR), Singapore, Singapore

4 National Cancer Centre Singapore, Singapore, Singapore