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RESEARCH

Prognostic Value of Ki-67 in Breast Carcinoma: Tissue Microarray Method Versus Whole Section Analysis- Potentials and Pitfalls

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Abstract In our study we have compared the prognostic value of two distinct methods of immunohistochemical Ki-67 determination, tissue microarray (TMA) and classical whole section analysis. "Cut-off" values were used according to the 2009 St. Gallen Consensus. Tissue specimens were obtained from a consecutive retrospective series of 215 female patients with primary invasive tumours. Two hundred and thirteen patients were included in the study. Data on Ki-67 was collected by both tissue microarray (TMA) and whole section analysis. Follow up data on overall (OS) and disease-free survival (DFS) were collected. Median follow-up was 95 months (range from 7.8 through 107 months). Mutual correlation of two Ki-67 determination methods was non-significant (Person's r=0.13417; p=0.0528). There was statistically significant association of whole section Ki-67 expression with histological and nuclear grade, progesterone receptor and HER2/neu status. The expression of Ki-67 protein in TMAs correlated only with histological and nuclear grade, but not with other traditional clinicopathological factors. Statistically significant differences in DFS (p=0.0156) and OS (p=0.0028) were confirmed between subgroups with low and high whole section Ki-67 expression. When subgroups with high and intermediate expression were compared, significant difference was found in DFS (p=0.0272), but not in OS (p=0.0624). On the other hand, there was no statistically significant difference either in DFS, or in OS, according to the expression of Ki-67 in TMAs (p=0.6529; p=0.7883; p=0.7966 for DFS, and p=0.8917; p=0.6448; p=0.4323 for OS, respectively). In our study, classical whole section was superior to TMA analysis in terms of prognosis and clinicopathological correlation. Our results indicate that the method used may have impact on prognostic significance of Ki-67. Further studies are needed, covering a greater number of patients and including a precisely defined stage and treatment patient cohorts, in order to solve controversies in Ki-67 assessment methodology.

Keywords Ki-67 · Breast cancer · Proliferation · Prognostic factor · Tissue microarrays

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Introduction

Ki-67 is a non-histone nuclear protein encoded by the MKI67 gene, expressed only in proliferating cells, but its exact role is still obscure. It was originally identified by Gerdes and colleagues in the early 1980s using a mouse monoclonal antibody against a nuclear antigen from a Hodgkin's lymphoma-derived cell line. This protein was named Ki after the researcher's location; Kiel University, Germany, with the 67 label referring to the clone number on the 96-well plate [1]. It has a role in the early steps of polymerase I dependent ribosomal RNA synthesis, but despite its presence in the cell division process, there are few published studies about its detailed functions [2, 3]. Two Ki-67 protein isoforms, weighted 345 and 395 kDa, have been identified.

Cells express the Ki-67 antigen during G1, S, G2, and M phases of the cell-cycle, but not during the resting phase G0 [4]. Therefore, this protein is used to measure the fraction of proliferating cells in a given cell population, since both normal and tumour cells express this protein [5]. Ki-67 expression varies throughout the different cell-cycle phases. It is low in the G1 and S phases and rises to its peak level in mitosis. Later in the mitotic phase, anaphase, and telophase, a sharp decrease in Ki-67 levels occurs [6]. Monoclonal antibody for this protein was developed in 1983. Initially, the antibody was used on fresh or frozen tissue. Over time, a different antibody called MIB-1 was assessed on paraffin sections after antigen retrieval [7]. A very good correlation was found between these two antibodies, with predominance of MIB-1 due to possibilities of retrospective analyses [8]. Healthy breast tissue expresses low levels of Ki-67 protein (less than 3 %) almost exclusively in cells which are oestrogen receptor (ER) negative. ER positive cells in normal premenopausal breast tissue do not proliferate and thus do not express Ki-67 [9]. However, in normal postmenopausal breast glands ER and Ki-67 co-expression can be found [10].

About 40 % of ductal carcinomas in situ (DCIS) express high levels of Ki-67 protein, correlating with higher tumour grade, comedo necroses, and, representing thus a good predictor of disease recurrence [11].

It has been shown that Ki-67 expression in tumour cells is a well-known measure of proliferation in breast cancer [9], correlating with histological grade, tumour size, nodal status, vascular invasion and thymidine labelling index [12], and reversely correlating with hormone receptor status [13]. Correlation between Her-2/neu status and Ki-67 expression is still not clear, with some studies confirming that correlation, while others demonstrating opposite results [14, 15].

MIB-1 antibody allows us immunohistochemical analysis of Ki-67 antigen expression in fixed tissues parallel with the determination of routinely used prognostic factors [16]. Despite the fact that it is an inexpensive, simple, and routinely used method, the existing guidelines of the American Society of Clinical Oncology (ASCO) do not include it in the list of required routine biological markers, mainly due to the lack of method standardization [17]. The first problem is discrepancy among studies in antigen assessing, staining procedures and cell counting. The second problem is the determination of a "cut-off" value for defining low and high risk subgroups in terms of prognosis [18, 19].

In our study we have compared the prognostic value of two distinct methods of immunohistochemical Ki-67 determination: tissue microarray (TMA) and classical whole section analysis. "Cut-off" values were used according to the 2009 St. Gallen Consensus [20].

Materials and Methods

Clinicopathological Characteristics of Breast Carcinoma Patients

Formalin–fixed, paraffin-embedded specimens of breast cancer were obtained from a consecutive retrospective series of 215 female patients with primary invasive tumours at the University Hospital Centre in Zagreb, Croatia, from September 2002 to September 2003. Ki-67 assessment was done by both methods for 213 patients. The majority of patients were older than 50, with the mean age being 57.6 years. Patients' age ranged from 30 to 87 years and the mean tumour diameter was 2.3 cm (Table 1). Annotated clinical follow-up information was available for 182 patients, so overall and disease-free survivals were done on this cohort of patients. The median follow-up was 95 months (range from 7.8 through 107 months). Patients initially underwent either modified radical mastectomy or lumpectomy with complete axillary lymph node dissection followed by radiation therapy of residual breast tissue. All of the lymph node-positive patients received adjuvant chemotherapy and/or hormonal therapy. Lymph

Table 1 Clinicopathological characteristics of breast carcinoma patients

Variable	Category	Number	%
Patient's age	≤ 50	72	33.49
	> 50	143	66.51
Postmenopausal status	NO	80	37.21
	YES	135	62.79
Tumour size	< 2 cm	119	55.35
	2–5 cm	85	39.53
	> 5 cm	11	5.12
Histological grade	1	33	15.35
	2	110	51.16
	3	72	33.49
Nuclear grade	1	19	8.84
	2	123	57.21
	3	73	33.95
Vascular invasion	-VE	201	93.49
	+VE	14	6.51
Nodal status	-VE	104	48.37
	+VE	74	34.42
	unknown	37	17.21
ER status	-VE	79	36.92
	+VE	135	63.08
PR status	-VE	104	48.60
	+VE	110	51.40
HER-2 status	1	33	15.35
	2	110	51.16
	3	72	33.49

node-negative patients received adjuvant chemotherapy only if adverse prognostic factors were present. Tumour samples and clinical information were obtained under Institutional Review Board approval. All histological slides were examined by one pathologist, and all samples were graded according to the Elston and Ellis grading scheme [21, 22]. For all patients, tumour size, histological type, histological and nuclear grade, steroid receptor status, involvement of axillary lymph nodes, HER-2 status, and lymphovascular invasion were obtained, as well as all treatment information. Immunohistochemistry for ER (H7096, Dako, Glostrup, Denmark), PR (M3569, Dako, Glostrup, Denmark), and HER2 (Herceptest, Dako, Glostrup, Denmark) was done on formalin-fixed, paraffin-embedded tissue slides with standard avidin-biotin-immunoperoxidase staining method using TechMate automatic stainer (Dako, Glostrup, Denmark). The evaluation of the staining results was similar to that used in routine diagnostics, and samples were considered positive when 10 % of the cells were stained with ER and PR. For HER2 status, tumours were considered positive if scored as 3+ according to Herceptest criteria. Dual SISH with amplification ratio of more than≥2.0 was used to segregate immunohistochemically equivocal (2+) results.

Tissue Microarray Preparation and Immunohistochemical Determination of Ki-67

Proliferation marker Ki-67 was obtained immunohistochemically in two ways: with TMAs and classical whole-section analysis [23]. For TMAs preparation the most representative area of the tumour was punched with a special needle (SAKURA, Japan) to produce a breast cancer tissue microarray including three cores (triplets), each 0.2 cm in diameter from original paraffin blocks. The cores were re-embedded into arrayed blank recipient blocks using a manual "arrayer" (Beecher Instruments, Sun Prairie, Wisconsin, USA). Using this method, 60 tumour samples were analyzed on one single slide. Five micrometer sections were cut from paraffin-embedded tissue microarray blocks, processed in xylene, and dehydrated in a series of graded alcohols. The sections were pre-treated with High pH 9.0 buffer (Dako, Glostrup, Denmark) for 20 min at 65 °C, 20 min at 98 °C, and 20 min at 65 °C and incubated with MIB-1 (Dako, Glostup, Denmark) antibody at 1:50 dilution at room temperature for 30 min. Staining procedures were done following the automated stainer standard protocol (DAKO autostainer Universal staining system, Denmark) using Envision-Flex Kit (Dako, Glostrup, Denmark) for 20 min. Antigen-antibody reactions were visualized with diaminobenzidine (DAB) as a brown nuclear staining on sections counterstained with haematoxylin. The same staining procedure for Ki-67 was also used on the whole sections of the original blocks. Samples of palatal tonsil were used as a positive control, and slides of breast cancer not incubated with primary antibody as a negative control. Staining for Ki-67 proliferation marker was presented as the percentage of positive nuclei per thousand tumour cells for both whole sections as well as tissue microarray triplets. Counting at so called "hot spots" was done, and

Table 2 Ki-67 index distribution in subgroups according to percentage of positive cells

Category (% of positive cells)	Ki-67 "whole-slide"		Tissue microarray Ki-67	
	Number	%	Number	%
<=15 %	144	67.6	168	79.1
15.1–30 %	35	16.5	26	12.1
30 %+	34	15.9	19	8.8

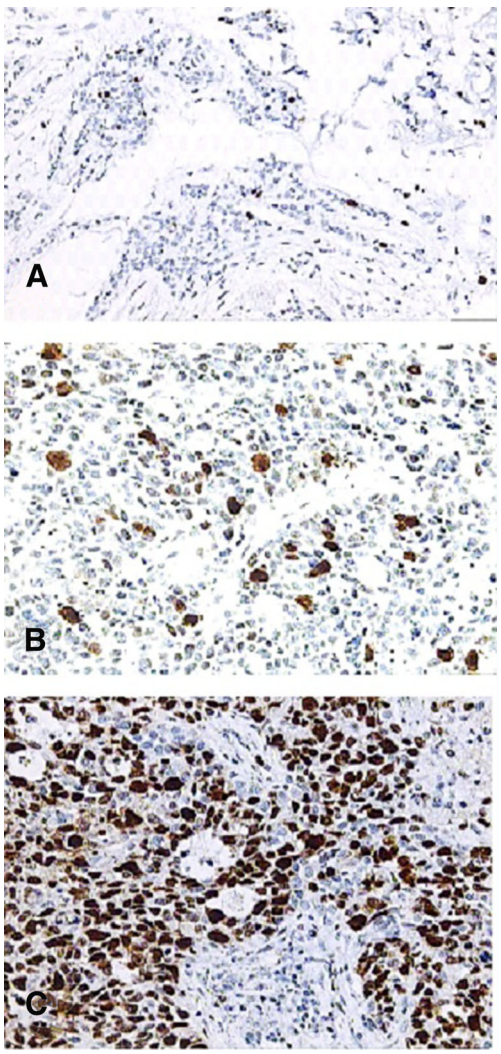

Fig. 1 Immunohistochemical staining with anti-Ki67 antibody (counterstaining haematoxylin) showing proliferating tumour cells: A. Less than 15 %. Counterstaining haematoxylin, × 200. B. Intermediate 15–30 %. Counterstaining haematoxylin, × 300. C. High Immunohistochemical staining with anti-Ki67 antibody showing proliferative index more than 15 %., × 400

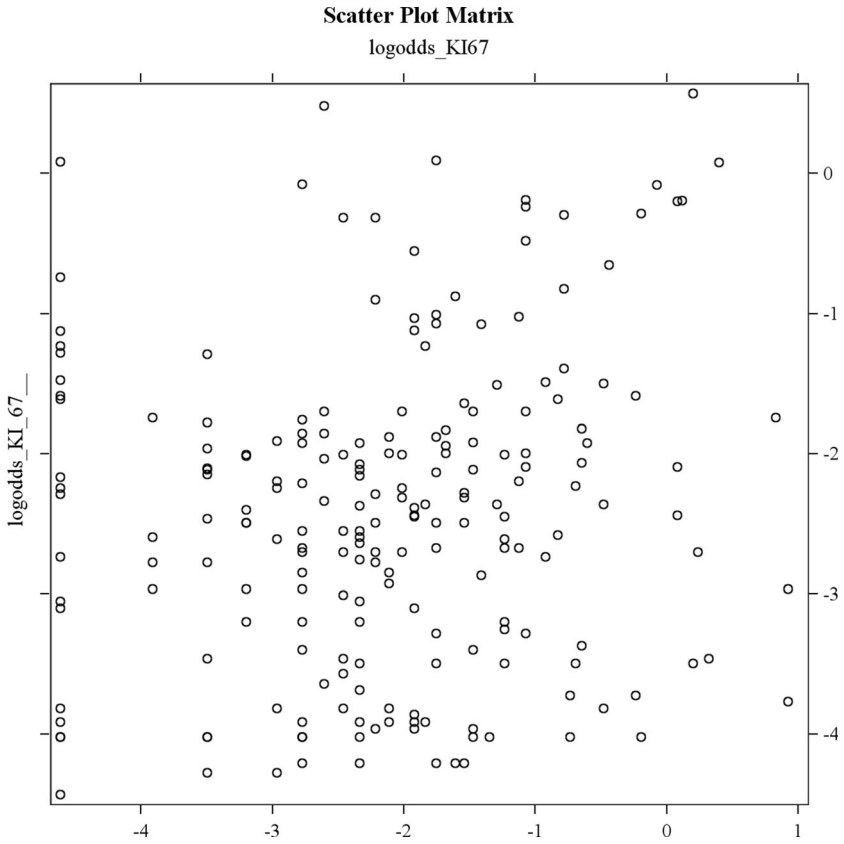

Fig. 2 Scatter-plot diagram of correlation between "whole-slide" Ki-67 (KI_67 on *y-axis*) and tissue microarray's Ki-67 (KI67 on *x-axis*): every single ringlet on the graph represent the point where each "whole-slide" and tissue microarray's Ki-67 value intersect (Pearson's correlation coeficient r=0.13417, p= 0.0528)

×400 objective magnification was used. Both analyses were done by the same experienced pathologist. Ki-67 index less than 15 % was considered to be low, 16 to 30 % intermediate and more than 30 % high [20].

Statistical Analysis

The percentage of Ki-67 positive cells was obtained for all patients with both methods, TMA and whole slide, expressed as continuous variables, and all values were log-odds transformed due to unequal variance. Parametric test (ANOVA) was used to analyze the correlations of proliferation markers with other clinicopathological variables, and Person's test for mutual correlations between the two Ki-67 index methods. The prognostic significance of Ki-67 index in whole slide and in TMAs was determined using a univariate Cox model. Univariate survival curves were generated by the Kaplan-Meier method and differences in survival were assessed by the log-rank test. Disease-free survival (DFS) and overall survival (OS) were used as end points. Statistical calculations were performed using SAS (SAS, Inc., Cary, NC) software. All of the tests of statistical significance were two-sided. P values <0.05 were regarded as statistically significant.

Results

Clinicopathological data of 215 breast carcinoma patients are shown in Table 1. Majority of the patients was older than 50, histological and nuclear grade II tumours, and tumours less than 2 cm in diameter. Table 2 shows Ki-67 index distribution in subgroups with "low", "intermediate" and "high" Ki-67 index, according to percentage of positive cells determined by TMAs and classical whole-section analysis method.

Table 3 Correlations of "classical" clinicopathological prognostic factors with "whole-slide" Ki-67 and tissue microarray's Ki-67 (*ANOVA*)

	Ki-67 "whole-slide"		Tissue microarray Ki-67	
	F	p	F	p
Histological grade	0.32390	<0.0001*	0.15727	0.0211*
Nuclear grade	0.27915	<0.0001*	0.15709	0.0212*
ER status	−0.09516	0.1716	−0.02004	0.7707
PR status	−0.15871	0.0220*	0.00694	0.9196
HER-2 status	0.20623	0.0027*	0.11220	0.1008
Tumour size	0.12023	0.0829	0.07520	0.2723
Patient's age	−0.02967	0.6698	−0.10836	0.1131
Nodal status	0.12560	0.0700	0.07633	0.2651
Menopausal status	−0.05185	0.4559	−0.04213	0.5389

*statisticaly significant

Table 4 Influence of Ki-67 on patients outcome (results of univariate analysis)

		Category	DFS *P* value	OS *P* value
Whole-slide Ki-67	<=15 %	15.1–30 %	0.8793	0.4555
	<=15 %	30 %+	0.0156*	0.0028*
	15.1–30 %	30 %+	0.0272*	0.0624
Tissue microarray Ki-67	<=15 %	15.1–30 %	0.6529	0.8917
	<=15 %	30 %+	0.7883	0.6448
	15.1–30 %	30 %+	0.7966	0.4323

*statisticaly significant

Immunohistochemical staining with anti-Ki67 antibody is shown in Fig. 1.

Mutual correlation of the two Ki-67 determination methods is shown in Fig. 2. Person's correlation test was used, with r= 0.13417 and p of 0.0528, which was non-significant, but near and slightly above statistically significant p value.

Association of Ki-67 Proliferation index with Other Clinicopathological Characteristics

Correlation of investigated Ki-67 proliferation index done by TMAs and whole section analysis and the well-known traditional prognostic factors were analyzed by ANOVA (Table 3). There was statistically significant association of whole section Ki-67 expression with histological and nuclear grade, progesterone receptor and HER2/neu status. We found a trend of positive association between whole section Ki-67 expression and both, the tumour size and nodal status of axilla (p= 0.0829 and p=0.0700, respectively). On the other hand, the Ki-67 expression in TMAs correlates only with histological and nuclear grade, but not with other traditional clinicopathological factors (ER status, PR status, HER-2 status, tumour size, patient's age, nodal and menopausal status).

Influence of Ki-67 Expression on Patients Outcome (Results of Univariate Analysis)

For overall (OS) and disease-free survival (DFS) analysis, the study population was divided into three subgroups: a) with high (more than 30 % positive cells), b) with moderate (16 to 30 % positive cells) and c) with low Ki-67 expression levels (15 % or less positive cells). The influence of Ki-67 expression on patient outcomes (DFS and OS) was determined using a univariate Cox model. The results of that univariate analysis are shown in Table 4. Statistically significant differences in DFS were confirmed between subgroups with low and high expression of Ki-67 on whole-section (p=0.0156), and

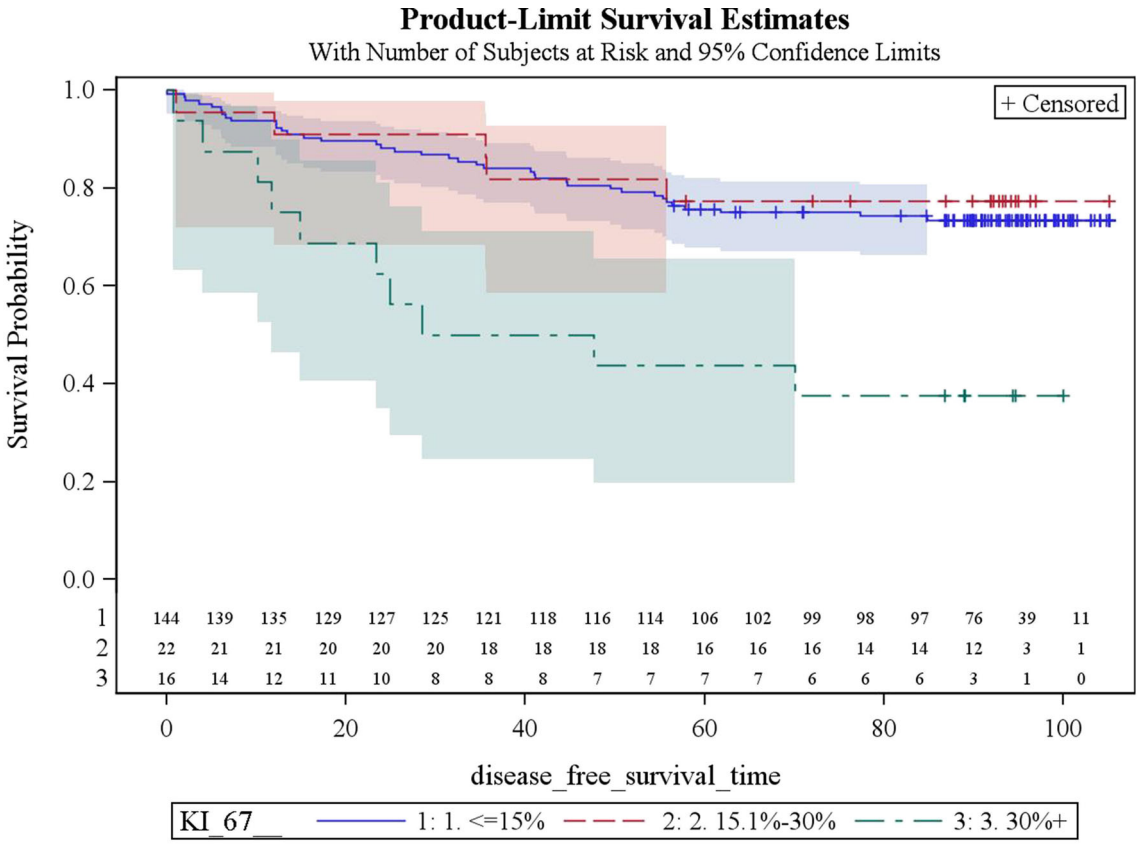

Fig. 3 Disease-free survival (*DFS*) according to whole-slide Ki-67 expression

between subgroups with high and intermediate expression, also (p=0.0272, Table 4, Fig. 3). With regard to overall survival (OS), statistically significant difference was found in the low Ki-67 expression subgroup in comparison with the high expression subgroup (p=0.0028) determined by whole-section Ki-67 analysis, while difference in OS between subgroups with high, intermediate, and low and intermediate whole-section Ki-67 expression was found to be near the statistical significance (p=0.0624; Table 4, Fig. 4). On the other hand, there was no statistically significant difference either in DFS, or in OS, according to the level of Ki-67 expression determined by TMAs (p=0.6529; p=0.7883; p= 0.7966 for DFS, Table 4, Fig. 5; and p=0.8917; p=0.6448; p= 0.4323 for OS, respectively, Table 4, Fig. 6).

Discussion

In the present study, the main question was whether the determination of Ki-67 status by TMAs could be regarded as equivalent to Ki-67 status determined by whole slides. Despite the fact that earlier studies showed that tumour cell proliferation could be reliably analyzed in a TMAs format, and could be reproduced with high statistical significance using a TMA containing only one tissue sample per tumour [24], our study has shown that classical whole section analysis over performs analysis of Ki-67 expression in TMAs. TMAs are an increasingly popular resource for assessing biomarkers, including Ki-67, used for analysis of outcome in large phase III clinical trials and epidemiological studies. There is some evidence that scores are generally lower in TMAs, but systematic comparisons of the Ki-67 assessment by TMAs vs. whole sections in breast cancer are still lacking. There is a recommendation that Ki-67 studies in TMAs should not be used for establishing cut-offs for clinical application on other types of samples.

Comparison of Ki-67 assessment in “core” biopsies vs. whole section showed higher scores in favour of whole section. International Ki-67 in Breast Cancer Working Group has, however, found both methods suitable [25].

Determination of Ki-67 expression in TMAs might also have technical difficulties because of its unequal intratumoural distribution (so-called hot spots). The assessment of Ki-67 by whole section could be done by several scoring approaches: hot spot scoring, inclusion of hot spots in general across the section scoring and by overall average score across whole section only. Although this scoring issue needs further clarification, the International Ki-67 in Breast Cancer Working Group recommends an overall average scoring method [25].

In Honma’s et al. study, the Ki-67 evaluation at the "hottest spot" was shown to be superior to that determined by average score across the section as a predictor of outcome in patients

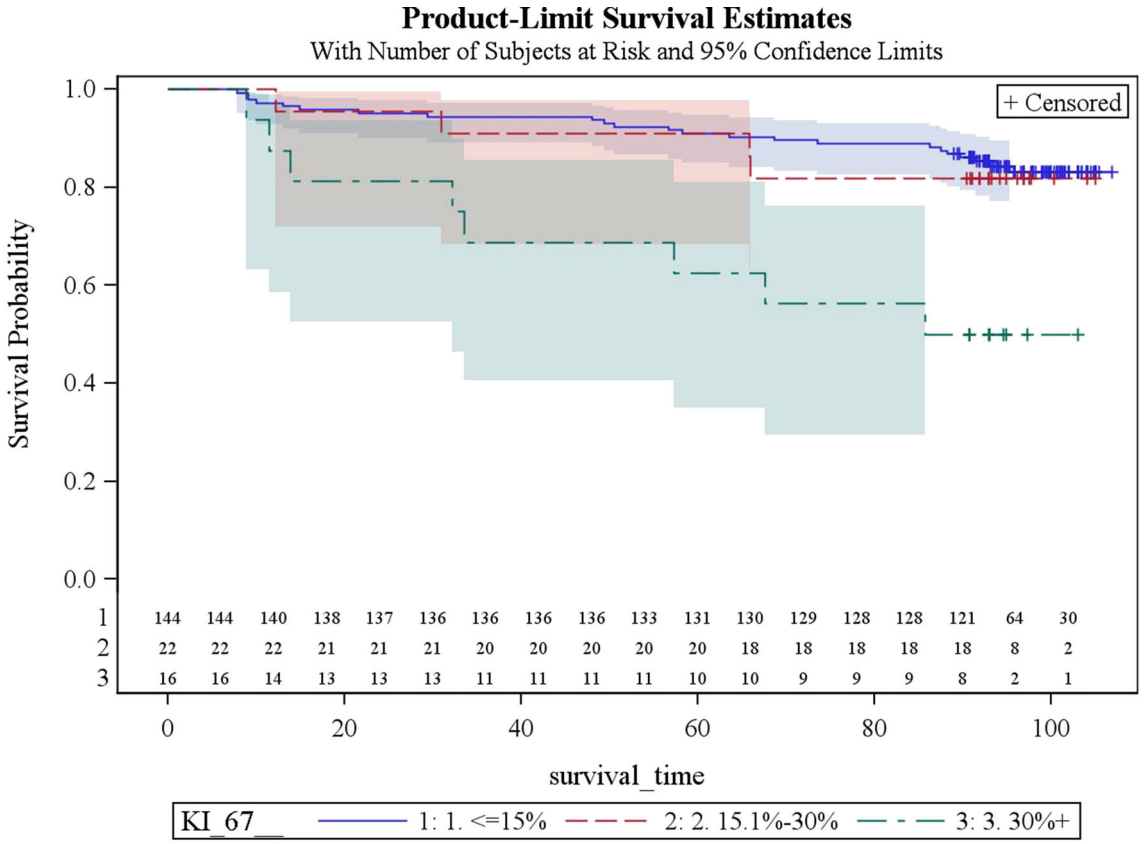

Fig. 4 Overal survival (*OS*) according to whole-slide Ki-67 expression

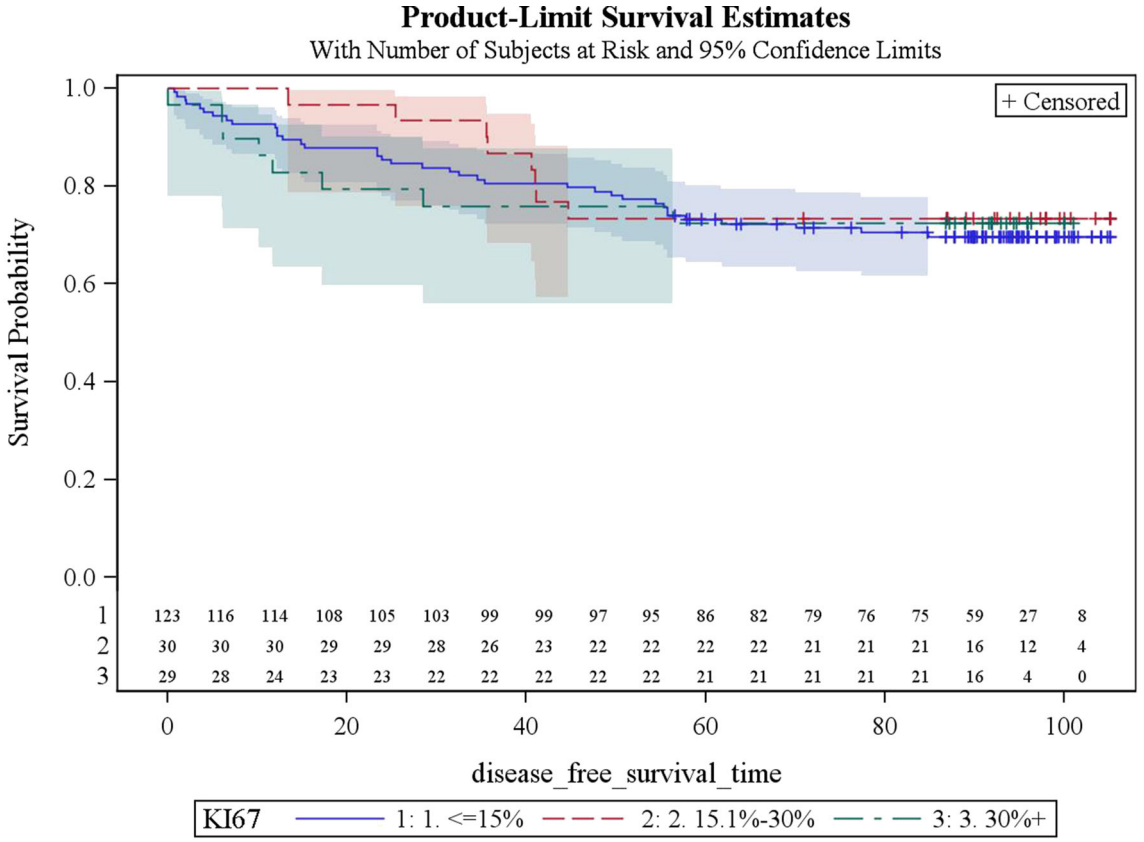

Fig. 5 Disease-free survival (DFS) according to tissue microarray's Ki-67 expression

with hormone receptor-positive/HER2-negative disease treated with tamoxifen. On the other hand, Ki-67 was not a predictor of clinical outcome in patients with triple-negative breast cancer in the same study [26].

The second problem, besides "hot spots", could be intratumoural heterogeneity, with a hindering effect on strategies that depend on results from tumour biopsy samples. Intratumour heterogeneity is a phenomenon recognized in oncology for decades [27]. Recently, genetic analyses provided additional data on this issue [28]. Besides contributing to the explanation of tumour therapy resistance, tumour heterogeneity also has impact on the field of biomarker validation.

In our study, 500 tumour cells were scored for each measurement. This is in accordance with other published studies, with 500 to 2,000 tumour cells scored. Our Ki-67 measurements have followed a log-normal distribution, which was concordant with most previous results [29].

Cut-off points used for distinguishing high from low Ki-67 index have been widely discussed in literature. It is not possible to apply general cut-off values to define tumours as having low, intermediate or high proliferative activity. Cut-off values may vary as a function of the antibody used and of the method of measurement (visual vs. automated scoring).

Computer assisted automated scoring can improve the accuracy and inter-observer reproducibility of Ki-67 assessments [30]. A group of authors compared automated versus visual counting method of Ki-67 status [31]. In their study, a cut-off value of 15 % was used, based on survival analysis for visually assessed Ki-67. The results of automated and visual assessment were in good agreement. However, automated Ki-67 assessment was inferior to the visual method in predicting breast cancer survival rate. Inter-observer differences are the reason why all analyses in our study were done by one experienced pathologist. Most data in the literature are derived from visual scoring. Image analysis computed methods remain to be proven for their use in clinical practice.

The most common cut-off values are based on the median value, values that discriminate best between subgroups with good and bad prognosis according to DFS and OS, or some arbitrary values usually between 10 and 20 %. Without the standardization of methodology, these cut-offs have limited value outside the studies from which they were derived.

In our study, the recommendations from the St. Gallen International Expert Consensus 2009 for adjuvant treatment of early breast cancer have been used including determination of tumour proliferative activity by Ki-67 index and number of mitosis as tumour proliferation markers. Depending on Ki-67 index, with less than 15 % being low, 16–30 % being intermediate and more than 30 % being high, ER positive patients will receive adjuvant chemotherapy along with hormonal

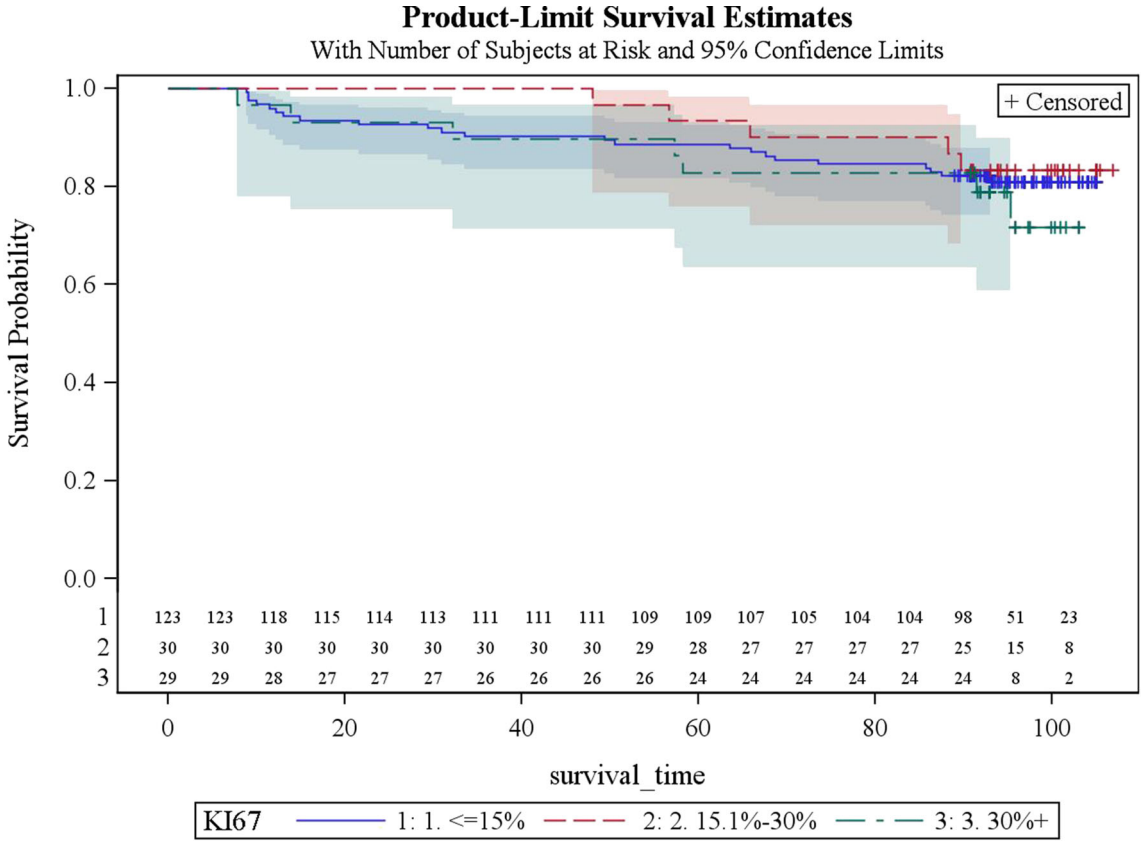

Fig. 6 Overal survival (OS) according to tissue microarray's Ki-67 expression

therapy [20]. Despite many cut-offs used, staining levels of 10–20 % have been the most common to dichotomize populations. Cheang and colleagues explored the role of oestrogen and progesterone receptor, HER2 protein, and Ki–67 index in distinction of subtype A and subtype B luminal breast cancer identified with 50 gene expression profiling. They determined luminal A subtype to have a low percentage of Ki-67 positive cells and subtype B to have a high percentage of Ki-67 positive cells, with cut-off point at 14 % [32].

Quantification of Ki-67 expression provides valuable information on tumour growth characteristics, sensitivity to different cytotoxic drugs, and risk of relapse [32]. Meta-analyses have tried to elucidate the prognostic and predictive roles of Ki-67 expression. The most important is Stuart-Harris and co-workers meta-analysis with forty-three studies analyzed [33]. Eleven of them have confirmed the prognostic value of Ki-67, while some smaller studies have not [34, 35]. The clinical utility of Ki-67 as a prognostic marker might be more apparent if considered as part of a multiparameter panel of biomarkers, for example IHC-4, which consists of oestrogen receptor (ER), progesterone receptor (PgR), HER2 and Ki-67 [36].

Retrospective analysis of risk factors for central nervous system metastases in primary operable breast cancers has shown that breast cancer with Ki-67 index equal to and above 30 % were associated with lower OS and DFS and higher cumulative incidence of CNS metastases compared with cancers with Ki-67 index less than 30 % [37].

The predictive role of Ki-67 on primary systemic treatment has been analyzed in few prospective and retrospective studies and has been found to be controversial. Penault-Llorca et al. have reported that high levels of Ki-67 were predictive of benefit from adding docetaxel to fluorouracil and epirubicin chemotherapy for patients with ER-positive tumours [38], while another study has found no predictive value [39].

There are indications that Ki-67 could be used to monitor the response to taxan therapy, but new studies are needed to determine its predictive value. Due to insufficient data on its predictive value [40], Ki-67 was omitted as a factor in deciding whether to use aromatase inhibitors instead of tamoxifen as adjuvant hormonal therapy in postmenopausal hormone-receptor positive patients [41]. Recent studies have shown promising results in using Ki-67 as a predictive factor for long-term remission after neo-adjuvant hormonal therapy [42].

The use of microarray tissue blocks makes it possible to stain all the samples at the same time and under the same conditions. Subgroups of the materials are therefore very well comparable, and a highly significant correlation between this kind of multicore system and studying the whole sections of the original blocks has also been shown [43, 44]. The selection

of the region of interest in tumours is another source of possible variability using the TMAs method.

Despite the greater diameter of each TMA triplet core in our study (2.0 mm vs. the usual 0.6 mm in the majority of other studies) whole section analysis has shown to be superior to TMAs in Ki-67 evaluation. It is concordant with the recommendation of the International Ki-67 in Breast Cancer Working Group, that TMAs are acceptable for clinical trial evaluation or epidemiological studies of Ki-67, but not for routine practice, until data of systematic comparison of the assessment of Ki-67 in TMAs vs. whole sections in breast cancer are published [25].

In conclusion, our study confirms significant relationship between Ki-67 assessed by whole section analysis and the survival of breast cancer patients. There are many potentials and pitfalls associated with Ki-67 as a cancer biomarker. Our study contributes to the evaluation of Ki-67 assessment with the aim of attaining reproducible methodology and consistent scoring methods. In our study, classical whole section was superior to TMAs analysis in terms of prognosis and clinicopathological correlation. Further studies are needed, covering a greater number of patients, and including a precisely defined stage and treatment patient cohorts, in order to obtain optimal clinical utility of Ki-67.

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Declaration of Interest The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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