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Do YOU know the Ki-67 index of your breast cancer patients? Knowledge of your institution's Ki-67 index distribution and its robustness is essential for decision-making in early breast cancer

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ABSTRACT

Objectives: The proliferative activity of the Ki-67 index is important in decision-making of adjuvant treatments in early breast cancer. Its reliability can be reduced by inter-observer variability. This analysis' objective is to evaluate the robustness of Ki-67 values within one center over 5 years and to compare its distribution with a published dataset.

Materials and methods: Ki-67 indices of early breast cancers treated at St. Gallen Breast Center were collected (2010–2014; 1154 patients). Distribution of Ki-67 values was analyzed for each year, along with histologic subtype and grading. Tumors were classified into intrinsic subtypes using two definitions: 2013 St. Gallen Consensus and the refined definition by Maisonneuve ("Milano Group"). Our institution's Ki-67 cut-off value was adjusted to obtain the same distribution of luminal subtypes as published data of the Milano Group.

Results: Ki-67 index frequency distributions were comparable between years (mean 26–30%, median 22–26%). Shape and position of the distribution curves were nearly identical. Ki-67 values correlated with tumor grade (median Ki-67: G1: 12.0%, G2: 21%, G3: 38%). Standard deviation of Ki-67 increased with higher grading (G1: 6.9; G2: 9.2; G3: 18.2; $p < 0.001$). According to the 2013 definition (and refined definition respectively), there were 35% (41%) luminal A-like and 65% (59%) luminal B-like tumors. To obtain the same distribution as the Milano group, Ki-67 cut-off needed to be elevated to 22%.

Conclusions: Ki-67 index assessment was comparable over many years. Knowledge of one's institution's Ki-67 value distribution is essential for clinical decision-making of adjuvant therapies in early breast cancer.

1. Introduction

Immunohistochemical (IHC) staining of the Ki-67 antigen has been used for many years as a marker for tumor proliferation. Potential uses include prognosis [1], prediction of relative responsiveness or resistance to chemotherapy [2] or endocrine therapy and as a dynamic biomarker of treatment efficacy of neoadjuvant therapy [3,4]. The proliferation rate is an important parameter to differentiate between luminal A and luminal B type of breast cancer.

For the first time, in 2005, the expert panel of the St. Gallen Consensus Conference For Early Breast Cancer recommended choosing adjuvant systemic therapy according to tumor biology rather than risk assessment only[5]. In 2007 the expert panel reaffirmed the primary importance of determining endocrine responsiveness of the cancer as a first step in the approach to selecting systemic therapy and determined three HER2-negative categories: highly endocrine responsive, incompletely endocrine responsive and endocrine non-responsive[6]. However, the threshold for the use of cytotoxic chemotherapy for patients with

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estrogen receptor-positive (ER-positive), HER2-negative disease has remained very difficult to define.

At the same time, the advent of microarray-based multigene assays has led to a new paradigm in deciphering breast cancer heterogeneity and several specific intrinsic subtypes have been described, including two prognostically different ER-positive subtypes, namely luminal A and luminal B subtype [7,8]. Hence several gene expression panels have been developed to determine the luminal subtype for predicting breast cancer prognosis and supporting the physicians in their recommendation for or against adjuvant chemotherapy [9,10].

However, gene expression profiling is still costly and not widely applicable in daily practise. Cheang et al. subtyped 357 tumors by gene expression and also determined immunohistochemically hormone receptor status, HER2-status and Ki-67-index[11]. Using receiver operating characteristic curves, a Ki-67 cut-off of 14% was determined to immunohistochemically distinguish between luminal B and luminal A tumors[11]. In 2011 the St. Gallen Consensus closely followed this proposal and stated a surrogate definition of intrinsic subtypes of breast cancer by choosing a Ki-67 cut-off value of 14% to differentiate immunohistochemically between luminal A-like and luminal B-like subtype [12].

In 2013, the St. Gallen Consensus Conference expert panel advised that the distinction between luminal A–like and luminal B–like tumors could be improved by including progesterone receptor (PgR) positivity ($\geq$20%) and by increasing the threshold of the Ki-67 index to $\geq$20% [13,14]. This change of criteria increased the number of patients classified as having luminal A–like breast cancer and thus decreased the number of patients for whom cytotoxic therapy is generally recommended. The panellists emphasized the importance of internal and external quality assurance programmes of pathology institutes and that Ki-67 value distribution should be known in order to allow adequate interpretation and assignment of cases to the luminal A-like or luminal B-like breast cancer subtype [14]. The expert panel in 2017 acknowledged that the classification based on routine histopathology was still clinically valuable and could be used for adjuvant treatment decisions[15]. Specifically, the panel agreed that either grading or Ki-67 could be used to distinguish between luminal A-like and B-like, but that gene expression signatures were preferable to standard pathology, when adequate reproducibility is not ascertained[15].

Ki-67 values have been shown to suffer from some inter-observer variability, especially in midrange (G2) breast cancer [16,17]. The cut-off values may vary considerably between different institutions. Therefore, it is essential that clinicians know the range of Ki-67 of their own pathology institute compared to microarray analysis or at least know their pathology institute's Ki-67 cut-off level to get similar proportions of luminal A-like and luminal-B like cohorts compared to the internationally published data.

The aim of this work was to deepen our understanding of Ki-67 in breast cancer as we use this value on a daily basis in clinical decision making. Our goal was to obtain information on robustness and thus reliability of our pathology institute's Ki-67 testing. Furthermore, we wanted to explore the relationship between Ki-67, grading and histologic subtype respectively. In the end, we compared our results with the distribution of Ki-67 values of the published data set of the Milano group[18] to determine where to set the cut-off value in our institution.

2. Material and methods

2.1. Patients

We retrospectively collected the data of patients with early breast cancer diagnosed between 2010 and 2014 in St. Gallen Breast Center, which comprises two hospitals (St. Gallen cantonal hospital and the smaller Grabs hospital, which is affiliated with St. Gallen cantonal hospital). Data of patients who had a Ki-67 assessment done at our institute of pathology but didn't receive treatment in St. Gallen Breast Center were not included. All Ki-67 assessments were included regardless of tissue type (biopsy or surgical specimen). In addition to Ki-67 values, we collected following parameters: stage (according to the TNM staging system, UICC 7th edition), histologic tumor type, tumor grade (according to the Bloom-Richardson-Elston system[19]), ER-, PgR-, HER2-status and type of tissue. We retrieved the information solely from the institute of pathology's database. In some cases, not all clinical parameters were available. We included only one Ki-67 value per patient. For those patients with multiple Ki-67 assessments available, the highest value was included in the analysis. For the calculation of the Ki-67 value distribution over the years, all patients were included. For the correlation of Ki-67 values with clinical markers we used only the subset of patients with all clinical data available.

2.2. Ki-67 staining

Ki-67 assessments were performed by the same two laboratory technicians over the whole study period. It was performed in a manual way for all cases. Two-μm thick formalin-fixed, paraffin-embedded tissue sections were deparaffinized followed by antigen retrieval (EDTA pH 9.0, 95 °C, 30 min). Ki-67 stainings were performed on a Leica BOND MAX instrument (Leica) using mouse monoclonal Ki-67 antibody (clone MIB1, dilution 1:80, 30 min; Dako) and the Bond Polymer Refine detection kit (Leica). Ki-67 index was defined as the percentage of invasive carcinoma cells with nuclear Ki-67 staining. For each tumor, at least 1′000 carcinoma cells in hot spot areas were scored. In the literature two ways of counting Ki-67 index are discussed: average counting and condensed proliferative areas, so called 'hot spot'- counting[20,21]. As in Switzerland the guidelines recommend counting hot spots we decided to follow this recommendation as it is traditionally done in our institution [21,22].

2.3. Comparison with the data set of the milano group

The Milano group classified the data of 9415 patients according to the definition of the Consensus Conference 2013 [14] into luminal A-like and luminal B-like subtypes, including all women who had undergone surgery for a first ER-positive, HER2-negative primary breast cancer at the Istituto Europeo di Milano (IEO) in Milan between 1994 and 2006 (Table 3, left) [18]. They separately analyzed the outcome of patients with intermediate Ki-67 levels (14–19%) according to the PgR expression (high $\geq$20% vs low <20%) and published evidence that patients with intermediate Ki-67 levels but substantial PgR positivity still had good outcomes compared to luminal A–like disease [18]. On the basis of this evidence, the Milano group re-defined the immunohistochemical classification of intrinsic subtypes (Table 3, right), optimizing the number of patients classified as having luminal A-like subtypes[18]. We compared our Ki-67 cut-off for immunohistochemical classification into luminal A-like and B-like with both definitions (St. Gallen Consensus 2013 and revised definition according to Milano group)[14,18]. In addition we determined the necessary cut-off to get the same proportion of luminal A-like and B-like tumors as the Milano group[18].

2.4. Statistical analysis

For the statistical analysis Microsoft® Excel® 2010, version 14

for Windows (Microsoft Corp., Redmond, WA) and IBM® SPSS® Statistics, version 20 for Windows (IBM Corp., Armonk, NY) were used. We tested for significance using Wilcoxon-Mann-Whitney-test (*U* test) when comparing two groups and Kruskal-Wallis-test (H-test) when comparing more than two groups. Significance level α was set at 5%, $p = 0.05$.

3. Results

3.1. Study population

Between 2010 and 2014, a total of 1154 patients with early breast cancer had a Ki-67 analysis done at our pathology institute. Most Ki-67 analyses (79%) were performed on surgical specimens.

The distribution of subtypes remained consistent over the years (Table 1).

The mean Ki-67 value of the entire cohort (n = 1154, all patients regardless of intrinsic subtype and completeness of additional data) was 28% (95%-CI [27.2%; 29.4%]). Ki-67 mean values varied in the range of 26–30%. Shape and position of the distribution curves of Ki-67 values did not differ between years (Kruskal-Wallis-Test, H-Test: $p = 0.18$)(Fig. 1, Table 2).

Mean Ki-67 values were significantly higher on biopsies than on surgical specimens (35.4% vs 26.6%, Wilcoxon-Mann-Whitney-Test, *U* Test: $p < 0.001$).

Distribution of luminal A-like and B-like tumors according to different definitions and comparison with the Milano data set.

According to the 2013 definition[14], our study population included 35% luminal A-like and 65% luminal B-like tumors (luminal B, HER2-type not included). According to the same definition the Milano group included 43% luminal A-like and 57% luminal B-like tumors in their data set[18]. To achieve a similar proportion of luminal A-like and luminal B-like tumors as in the Milano group, the Ki-67 cut-off value would have to be set at 22% at our institution.

According to the revised definition of 2015 the proportion of the Milano data set shifts to 52% luminal A-like and 48% luminal B-like tumors[18]. Using the revised definition in our study population 41% were classified as luminal A-like and 59% as luminal B-like tumors (luminal B, HER2-type not included). To reach the same distribution between luminal A-like and luminal B-like tumors as the Milano group, a Ki-67 cut-off value of again 22% would be necessary at our institution.

Variation of Ki-67 values according to luminal type, histology and grading (luminal cohort only).

For the correlation of Ki-67 values with clinical markers we used the subset of all patients with a luminal type (ER+/HER2-) tumor (n = 667) and all clinical data completely available (Table 4).

Looking at the variation of Ki-67 values for luminal A-like and B-like tumors (using the definition of 2013 [14]), it is evident, that there is significantly more variability in the group of luminal B-like tumors. (Standard deviation of luminal A-like 4,8; standard deviation of luminal B-like 15.1) As expected, this difference in heterogeneity was statistically significant (Wilcoxon-Mann-Whitney-Test, *U* Test: $p < 0.001$). A low Ki-67 value however does not rule out a luminal B-like tumor when applying the additional criterium of negative or low PgR (Fig. 2A).

In our population there were 511 (77%) ductal and 132 (20%) lobular cancer and 24 (3%) special types. The Ki-67 of lobular cancer was lower (median 18% vs 22%) and showed a narrower range compared to ductal cancer (standard deviation 15.7% vs 9.6%) and did usually not exceed 45% (Fig. 2B).

Ki-67 values correlated with tumor grade, with higher grades having higher Ki-67 values. G1-tumors had a median Ki-67 of 12.0%, G2-tumors 21% and G3-tumors 38%. The standard deviation of Ki-67 increases with higher grading. (G1: 6.9; G2: 9.2; G3: 18.2). The differences in distributions were highly significant (Kruskal-Wallis-Test, H-Test: $p < 0.001$). G3-tumors with a Ki-67 below 20% were extremely rare. On the other hand, G1-tumors tended to have Ki-67 values below 20% (Fig. 2C).

4. Discussion

The aim of our study was to investigate reproducibility and thus reliability of Ki-67 values over a 5-year-period at our institution and to determine a local Ki-67 cut off value to differentiate between luminal A-like and B-like tumors. The consistency of Ki-67 value distributions over the examined five years was remarkable (Fig. 1). The robustness of both median Ki-67 values and frequency distributions together with small confidence intervals indicate that a high degree of robustness can be achieved, if a standardized approach for Ki-67 index measurement is applied. Knowledge of the Ki-67 histograms (position and shape) is essential to make an adequate clinical interpretation of a reported Ki-67 result. Our results compare well with a published quality control study on the yearly distribution of predictive factors in early breast cancer[23]. This study did not report a significant yearly variation in mean or median Ki-67 values either and interpreted this as a reliable quality reassurance result for constant performance[23].

To determine the cut-off value for differentiating into luminal A-like and B-like tumors, we could have run multigene expression panels on all specimens and then compared the results with immunohistochemically determined intrinsic subtypes. Due to the high costs of this approach, we decided to compare our data set with the larger data set of the Milano group[18]. They correlated their definition of intrinsic subtypes with outcome-data and could therefore show the accuracy of their definition. The Milano group published their distribution in luminal A-like and B-like tumors according to two different definitions, 2013 and 2015, respectively, both with a Ki-67 cut-off value of 20%[18]. To get a similar distribution in our population and with our pathology institute, we needed a cut-off value of 22% for both definitions. It seems that for our institution 22% rather than 20% is the best Ki-67 cut-off for the classification into luminal A- and B-like tumors. We compared two different patient collectives, bearing the risk of drawing unjustified parallels. However, we chose the Milano data set[18], because both

Table 1
Distribution of intrinsic subtypes for each year and for the whole period (2010–2014). Classification into luminal-type (ER-positive, HER2-positive not included), HER2-type and triple-negative breast cancer according to the 2013 St. Gallen Consensus definition).

	2010–2014	2010	2011	2012	2013	2014
No. of patients [n]	1154	178	222	247	254	253
Luminal-type [n (%)]	873 (75.6)	128 (71.9)	167 (75.2)	188 (76.1)	203 (79.9)	187 (73.9)
HER2-type [n (%)]	148 (12.8)	16 (8.9)	33 (14.8)	37 (15.0)	30 (11.8)	32 (12.7)
Triple negative [n (%)]	67 (5.8)	15 (8.4)	12 (5.4)	12 (4.9)	9 (3.5)	19 (7.5)
Other [n (%)]	3 (0.3)	1 (0.6)	0 (0.0)	1 (0.4)	1 (0.4)	0 (0.0)
Unknown (ER/PgR/HER2) [n (%)]	63 (5.5)	18 (10.1)	10 (4.5)	9 (3.6)	11 (4.3)	15 (5.9)

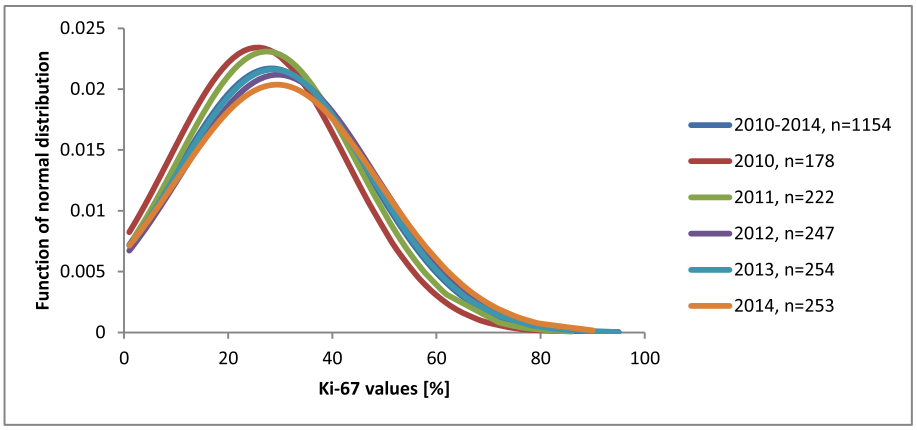


Fig. 1. Frequency distributions of Ki-67 values for each year and for the whole period (2010–2014).

Table 2
Ki-67 values for each year and for the whole period (2010–2014).

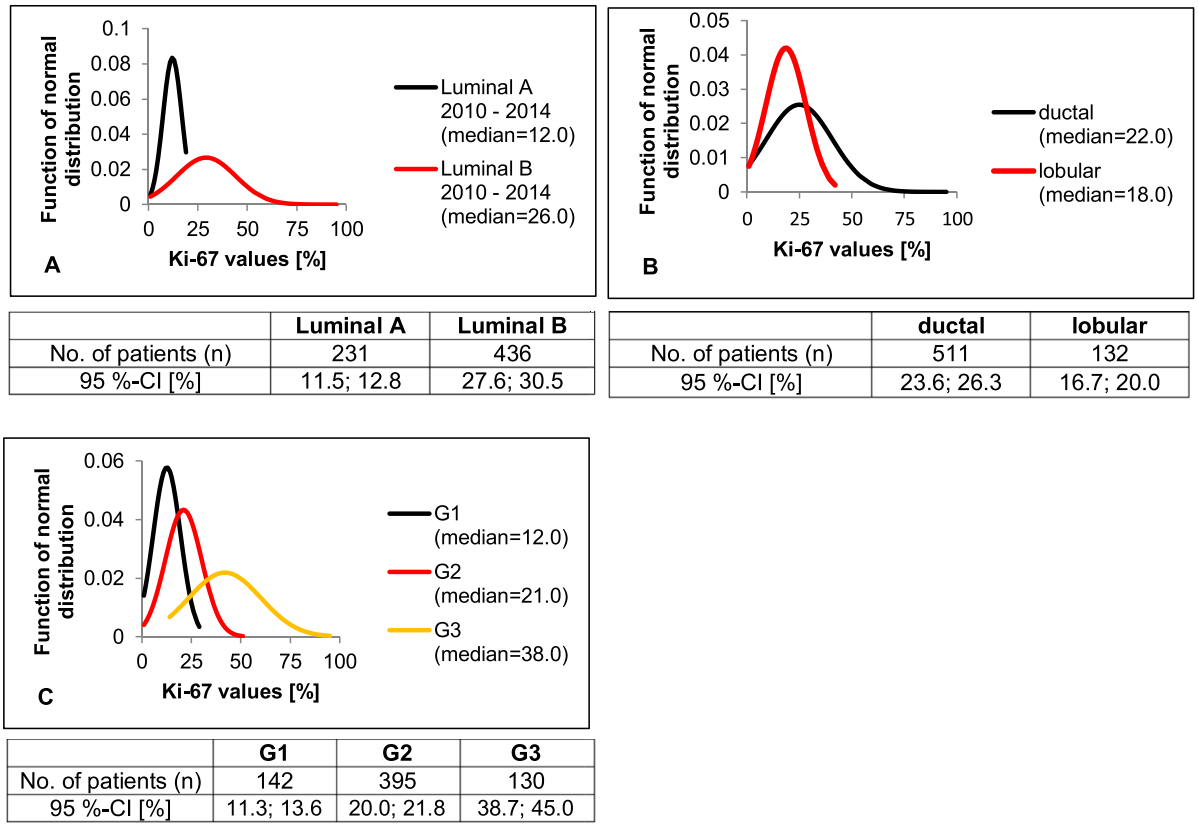
Ki-67 index [%]	2010–2014 (n = 1154)	2010 (n = 178)	2011 (n = 222)	2012 (n = 247)	2013 (n = 254)	2014 (n = 253)
Median	24	22	23	26	25	25
Mean	28	26	27	30	29	29
Standard deviation	18	17	17	19	19	20
95% confidence interval	27; 29	23; 28	25; 30	27; 32	26; 31	27; 32

Table 3
Definition of the intrinsic subtypes luminal A-like and luminal B-like according to the 2013 St. Gallen Consensus[14] (on the left) and the definition by Maisonneuve et al. [18](to the right).

	Definition St. Gallen Consensus Conference 2013 [14]	Revised definition according to the Milano group [18]
Luminal A	**"Luminal A"** - ER and PgR positive - HER2 negative - Ki-67 low (<20%)	**"Luminal A"** - ER positive - HER2 negative - *And either* - Ki-67 low (<14%) - *Or* - Ki-67 intermediate (14–19%) and PgR high (≥20%)
Luminal B	**"Luminal B (HER2 negative)"** - ER positive - HER2 negative - And at least one of the following: - PgR negative or low - Ki-67 high **"Luminal B, HER2-Typ (HER2 positive)"** - ER positive - HER2 positive - any Ki-67 - any PgR	**"Luminal B (HER2 negative)"** - ER positive - HER2 negative - *Either:* - Ki-67 intermediate (14–19%) and PgR low or negative (<20%) - *Or:* - Ki-67 high (≥20%)

Table 4
Tumor characteristics of luminal tumors (ER+/HER2-) for each year and for the whole period (2010–2014).

	2010–2014	2010	2011	2012	2013	2014
No. of patients [n]	667	104	136	144	155	128
pT0 [n (%)]	2 (0.3)	0 (0.0)	0 (0.0)	1 (0.7)	0 (0.0)	1 (0.8)
pTis [n (%)]	3 (0.5)	0 (0.0)	0 (0.0)	1 (0.7)	1 (0.6)	1 (0.8)
pT1 [n (%)]	365 (54.7)	57 (54.8)	77 (56.6)	75 (52.1)	82 (53.0)	74 (57.8)
pT2 [n (%)]	263 (39.4)	43 (41.3)	49 (36.0)	62 (43.0)	63 (40.6)	46 (35.9)
pT3 [n (%)]	30 (4.5)	3 (2.9)	10 (7.4)	3 (2.1)	8 (5.2)	6 (4.7)
pT4 [n (%)]	4 (0.6)	1 (1.0)	0 (0.0)	2 (1.4)	1 (0.6)	0 (0.0)
pN0 [n (%)]	391 (58.6)	52 (50.0)	79 (58.1)	86 (59.7)	88 (56.8)	86 (67.2)
pN+ [n (%)]	276 (41.4)	52 (50.0)	57 (41.9)	58 (40.3)	67 (43.2)	42 (32.8)



	Luminal A	Luminal B
No. of patients (n)	231	436
95 %-CI [%]	11.5; 12.8	27.6; 30.5

	ductal	lobular
No. of patients (n)	511	132
95 %-CI [%]	23.6; 26.3	16.7; 20.0

	G1	G2	G3
No. of patients (n)	142	395	130
95 %-CI [%]	11.3; 13.6	20.0; 21.8	38.7; 45.0

Fig. 2. **A:**Distribution of ki-67 according to luminal type (using the 2013 st. Gallen consensus definition) **B:**Distribution of ki-67 according to histological type. **C:** Distribution of ki-67 according to tumor grading.

collectives represent general breast cancer centers located in Europe. Both centers are referral-centers for breast cancer patients and both data sets comprised all patients who had undergone surgery for early breast cancer. Comparing the two data sets, it became evident, that only a slight adjustment of our Ki-67 cut-off was necessary to obtain a similar proportion of luminal A-like versus luminal B-like subtypes as the Milano group.

We also analyzed the correlation between Ki-67 values and histopathologic variables (grade, histologic subtype). Higher tumor grade was correlated with higher Ki-67 values and larger standard deviations. G3-tumors with a Ki-67 below 20% were extremely rare. On the other hand, G1-tumors tended to have Ki-67 values below 20%. However, there were some G1-tumors with high Ki-67 values which thus were classified as luminal B-like tumors. In this case it is unclear which value (grading vs Ki-67) is more reliable regarding prognosis. In a Japanese cohort of ER+/Her2-tumors, discordance with low histologic grade and high Ki-67 values were relatively frequently observed in 3 histologic subtypes: papillotubular invasive ductal carcinoma, invasive ductal carcinoma with predominantly intraductal component and mucinous carcinoma[24]. The latter two are considered as low-risk group of tumors for which only adjuvant endocrine therapy is recommended[24]. In our population, G2-tumors had a median Ki-67 of 21% and therefore comprised both luminal A- and B-like types. The 2017 St. Gallen Consensus agreement states, that either grading or Ki-67 can be used to distinguish between luminal A- and B-like tumors[15]. In above mentioned Japanese cohort in which outcome data was analyzed according to histologic grade, G1 und G2-tumors showed a good breast cancer specific survival with almost overlapping curves[24]. G3-tumors had a worse outcome with the curve separating clearly from G1 and G2-curves and the authors therefore concluded, that it was reasonable to consider withdrawal of adjuvant chemotherapy in node-negative invasive histologic grade 2 tumors[24]. Another problem with Ki-67 assessment of moderately differentiated tumors is, that they show a higher inter- and intra-observer variability compared to the assessment of Ki-67 in G1- and G3-tumors[16]. Therefore, Ki-67 values which are not clearly higher or lower than the cut-off value might have to be interpreted cautiously in G2-tumors. However similar observations were made for gene signatures. G2-tumors are most likely to show intermediate risk (depending mainly on cut-off of the lower threshold separating low from intermediate risk)[25].

The question arises, whether there is a place for conventional pathological subtyping (based on IHC including Ki-67) in the times of genomic testing. Concordance between pathological subtyping and molecular subtyping are reported to be low, mainly due to unreliable Ki-67 values and grading[26]. However, genomic testing has the downside of considerable costs and in some areas limited availability. Nomograms to predict recurrence score results have been developed[27]. Using clinicopathological and immunohistochemical variables (e.g. Ki-67), the low recurrence score subgroup can be predicted accurately[27]. Furthermore IHC4[28] results in comparable prognostic information when compared to gene-signatures [29]. In daily practice, oncologists often order genomic testing when the determination between a luminal A- or luminal B-like tumor is difficult using clinical information only (mostly G2 and/or Ki-67 around the cut-off value). However, in a considerable number of these cases, the result is, depending on the test, either an intermediate score[25] or a score close to the dividing line.

Interestingly, we saw clearly lower median Ki-67 values for

lobular than for ductal cancer (18% vs 22%) with almost no values above 50% for lobular cancer. Consequently, the percentage of luminal A-like tumors was higher for lobular subtypes than for ductal subtypes (39% vs 26%), these findings correlate with published characterization of lobular cancer[30,31].

There are reports about discrepancy between Ki-67 values assessed on core biopsies and surgical specimens, with some of them observing higher Ki-67 values on core biopsies [32,33] while others reported the opposite [34]. In the period of 2010–2014, in our institution Ki-67 was not routinely assessed on the initial biopsy, but was later done on the surgical specimen. Ki-67 assessments were only performed on the initial biopsy on request, e.g. if the other factors suggested an aggressive carcinoma and additional information to justify a neoadjuvant chemotherapy was sought. Therefore it is not clear, whether an observed difference in Ki-67 values would be due to a real difference or would be a mere expression of a selection bias.

5. Conclusion

In regard to breast cancer, Ki-67 is a valuable marker, as it has prognostic and predictive abilities. Its main problem is the limited reproducibility due to lacking standardization of procedures and inter-observer variability. We have shown that standardization of procedures and reducing inter-observer variability by limiting the number of observers results in surprisingly high reproducibility over years and thus robustness of results. Comparing an institute's Ki-67 values along with the proportion of luminal A-like versus luminal B-like subtypes to a data set with comparable characteristics can help to define the institute's cut-off value. Due to the low cost of testing and widespread availability, Ki-67, properly assessed, maintains an important position in the daily practice of breast cancer centers.

Declaration of competing interest

No conflicts of interest of any author.

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