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Different methods of pretreatment Ki-67 labeling evaluation and the prediction of breast cancer regression following neoadjuvant chemotherapy

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Abstract

Increased proliferation activity of breast cancer cells evaluated by Ki-67 immunohistochemistry, i.e. a high Ki-67 labeling index (Ki-67 LI), may predict better tumor regression in case of neoadjuvant chemotherapy. Despite recommendations for the evaluation of Ki-67 LI, there are variations in methodology.

We assessed the effect of different evaluation methods on the Ki-67 LI in patients with different response to neoadjuvant chemotherapy.

Thirty pretreatment core-biopsy samples of patients receiving neoadjuvant docetaxel-epirubicin chemotherapy with or without capecitabine were evaluated for their Ki-67 LI. Pathologic regression was categorized as no regression, partial regression and complete regression, with 10 cases in each category. Three antibodies (MIB1, B56, SP6), 4 observers and 4 methods (counting or estimating on glass slides and counting or estimating on representative digital images) were compared. The Kruskal-Wallis test and analyses of variance were performed to investigate the differences in Ki-67 LIs between different clinical outcomes (tumor regression categories).

Breast carcinomas with pathological complete regression had a higher mean Ki-67 LI than tumors not achieving complete regression with any methods, observers and antibodies investigated, although there was a variation between different evaluations in what may represent high proliferation. Estimating the Ki-67 LI on digital images representing the highest proliferation in the core biopsy seemed the best in separating complete responders from non-responders.

High Ki-67 LI values may predict pathological complete regression independently of the method of evaluation used, although the definition of high proliferation is problematic. Estimating the Ki-67 LI may be an adequate method of evaluation.

Key words: breast carcinoma, evaluation methods, Ki-67, neoadjuvant therapy, tumor regression

Introduction

Proliferation activity is of prognostic importance in breast cancer and the higher the proliferation, the faster the progression of the disease [1-4]. One component of the classical grading system is counting the mitotic figures in 10 high power fields [5]. Many other techniques are available to assess tumor cell proliferation beside mitotic counts, including flow cytometry, which examines the proportion of cells being in the S phase of the cell cycle, determination of thymidine-labeling index, or immunohistochemistry with antibodies to proliferating cell nuclear antigen (PCNA), or cyclins E and D [6]. However Ki-67 immunostaining is the most popular in current everyday practice. The reasons for this are the simplicity of the method and the fact, that this protein is expressed during the whole cell cycle except the G0 phase, therefore nuclear staining is present in all cells in the cycle [7,8]. Although cells in the cycle may reflect the proliferative fraction, some of the cells may also undergo apoptosis rather than mitosis.

Expression of this protein has not only prognostic value, but may also be predictive of the response to neoadjuvant therapy as suspected by Fasching et al. [9] and by Yerushalmi et al. [4], who analyzed the results of different studies looking for the predictive value of Ki-67 labeling index (LI: i.e. the proportion of Ki-67 stained tumor cells expressed as a percentage) in case of neoadjuvant chemotherapy. In these studies, better response (complete pathological response / pCR or complete clinical response / cCR) has been reported in breast cancers with higher proliferative activity treated with neoadjuvant chemotherapy [10−15]. Luprosi et al. also found that pCR is in connection with high Ki-67 LI although the predictive value of this marker was not verified [16]. Recent recommendations (including the St. Gallen International Breast Cancer Conference, 2013) contain Ki-67 evaluation as the part of the routine diagnostic procedures although its predictive potential has not yet been proven unequivocally in case of neoadjuvant chemotherapy [17−20].

During Ki-67 immunohistochemical examinations, the proportion of positive (stained) cancer cells is determined in percentage. This evaluation is based on the pathologist's own decision. Some pathologists use estimation, while others count the positive and negative cells in an area with a certain size or average the values of many different areas. Several published recommendations exist regarding the evaluation techniques of Ki-67, however there is no uniformly accepted one [16-18, 21, 22]. It is also notable that different detailed counting methods (e.g. counting 500 or 1000 tumor cells) are time consuming and can be difficult to use in the everyday practice of centers managing significant number of patients. In our previous studies we found that different evaluation methods have different reproducibility [23]. Our previous results also support the use of representative digital images in combination with visual evaluation as they increased the reproducibility of Ki-67 assessment [24]. Reproducibility is an important factor, as it may contribute to the predictive value of Ki-67. In our previous studies we analyzed only the reproducibility of Ki-67 evaluation on core-biopsy samples of patients who underwent neoadjuvant chemotherapy on the basis of their locally advanced stage. We also had the information on the therapeutic response, as the group originally studied included equal numbers of cases from each of the pathological complete response, partial response and no response categories.

The aim of the present study was to compare different therapeutic response categories with different Ki-67 evaluation methods in the cases previously analyzed for reproducibility [23, 24]. We intended to know if there was an evaluation method (among the previously used ones) which could be particularly recommended or rejected in everyday practice. Another important issue is the usability of the cut-off values in the different recommendations assorting breast carcinomas into low- and high proliferation groups [16-18]. In the present paper we also aimed to know whether we could identify a cut-off value which can be considered as separation point between tumors having high or low proliferation activity before neoadjuvant therapy and is worth of further studies.

Materials and methods

Thirty core-biopsy samples of patients with operable T2≥3 cm or T3-4 and/or N1-2 and M0 breast cancer candidate for neoadjuvant docetaxel-epirubicin chemotherapy with or without capecitabine [25] were retrospectively analyzed for their Ki-67 LI. Core-biopsy samples were chosen by considering regression of the primary tumor after neoadjuvant chemotherapy. Three categories were made: no regression (or progression), partial regression (histological signs of regression) and pathological complete regression (no residual invasive tumor). Each group consisted of 10 samples.

Samples were immunostained for Ki-67 with the following 3 antibodies: SP6 (monoclonal rabbit antibody, Hisztopatologia Kft., Pécs, Hungary), B56 (monoclonal mouse antibody, Hisztopatologia Kft., Pécs, Hungary) and MIB-1 (monoclonal mouse antibody, Dako, Glostrup, Denmark). Four different investigational methods were used to evaluate the Ki-67 LI.

In Method 1 (counting on glass slides) all samples were assessed independently by 3 pathologists at high-power magnification (X400). Each pathologist was asked to evaluate the slides according to his/her daily routine practice. The ratio of Ki-67 positive cells was evaluated with 5% accuracy. Each participant, following daily practice, evaluated the LI on the basis of one hundred tumor cells. Owing to the 5% precision, only values ending with 5 or 0 were recorded. To avoid bias arising from remembering the LI of a given sample, the observers first assessed all cases stained with one antibody and this was followed by the evaluation of all cases stained with the second antibody and finally the cases stained with the third antibody were analyzed [23].

In another study, four investigators analyzed representative areas of the same 30 samples [24]. Microphotographs were taken of each immunostained core biopsy sample at the same magnification (200X). The hot-spot area was photographed in all cases where such a hot spot could be identified. A single digital photo of each biopsy sample was selected for the study, and the pictures were entered in a Microsoft PowerPoint file. In this study, Method 2 was applied (Counting on digital images): a uniform grid composed of equidistant parallel horizontal lines was laid on all digital images (Figure 1), previously used for estimation. The observers were asked to count the tumor cells crossed by the lines or touching the lines. The lines of such a grid can be followed and the touching or crossed cells can be recorded (counted) continuously without the doubt of double counting or omitting single cells. Both immunohistochemically negative and positive nuclei were counted. Non-cancerous cells (stromal elements, lymphocytes etc.) were ignored as much as possible. The ratio of positive cells was derived from these values.

Method 3 (Estimation on digital images) was also used in this second study: before counting the proportion of Ki-67 stained cells with the help of the overlaid grid, investigators determined the Ki-67 LI by estimating the proportion of stained cells with 5% accuracy in the same areas (i.e. the same digital image displayed on a screen without the grid). No counting was involved in this assessment.

For the present study, the previously mentioned three methods were completed with a fourth, in which the proportion of Ki-67 labeled cells was estimated by eyeballing on the glass slides (Method 4). The same four investigators estimated the proportion of positive tumor cells on the slides by quick inspection of the slides, without counting.

The average Ki-67 LIs of the respective 10 cases of all observers, antibodies and assessment methods were used for comparisons of clinical outcomes (tumor regression categories). The Kruskal-Wallis test and analyses of variance (one-way and two-way ANOVA) were performed to investigate the differences in Ki-67 LIs between different clinical outcomes. Receiver operating characteristic (ROC) curve analyses were applied to broadly approach a distinction between Ki-67 values of cases with complete response to therapy and those without it. All analyses were performed with the statistical software package SPSS 15.0 for Windows (SPSS Inc., Chicago, Illinois).

Because of the retrospective nature of the study, anonymous handling of the available data and the lack of influence on patient outcome, no ethical approval was deemed necessary according to local regulations. The institutional data safety monitor (Bács-Kiskun County Teaching Hospital) was consulted and made no objections to the anonymous handling of the data.

Results

Altogether 1350 evaluations were analyzed in our study (as one investigator did not take part in the first count, i.e. Method 1). The overall mean value of the Ki-67 LI (with all assessment methods, antibodies and observers considered) was 54.22 (95% CI: 53.13-55.31).

According to the statistical analyses, values of Ki-67 LI were significantly different in the different regression groups: values of the group without regression were significantly lower than the values of the group showing complete regression ($p<0.0001$). The mean values of Ki-67 LI taking into account all methods of assessment were 66.61 (95% CI: 64.71-68.50), 51.32 (95% CI: 49.43-53.21) and 44.72 (95% CI: 42.83-46.62) for the groups with complete, partial and no regression, respectively.

The two way analysis of variance using the methods of evaluation and the response categories as factors showed significant differences in mean Ki-67 values according to both factors. The mean KI-67 value was the lowest in Method 1 and highest in Method 3. Mean Ki-67 LI values were 46.63 (95% CI: 44.21-49.05), 54.62 (95% CI: 52.52-56.72), 61.59 (95% CI: 59.49-63.69) and 54.04 (95% CI: 51.94-56.17) in case of Methods 1 through 4, respectively.

We found similar results after taking the pathologically assessed response into consideration. Mean value of Ki-67 LIs by Method 1 were 58.72 (95% CI: 54.53-62.92), 46.39 (95% CI: 42.19-50.59) and 34.78 (95% CI: 30.58-38.98) in case of complete, partial and no regression, respectively, whereas these values with Method 3 were 77.18 (95% CI: 73.54-80.18), 56.59 (95% CI: 52.95-60.13) and 51.00 (95% CI: 47.37-54.64), respectively.

Figure 2 remarkably illustrates that the red and blue fields representing the mean Ki-67 LIs of cases with pCR and no regression, respectively are best separated from each other in case of Method 3, the estimation on single digital images and this is in accordance with the mean Ki-67 LIs described above. It is also clearly demonstrated that the yellow fields, which show partial regression are mostly located between the red and blue fields, slightly closer to the blue ones; sometimes they are fused with them (green). These patterns can be observed in case of all investigational methods, observers and antibodies.

By applying an ROC curve analysis to the mean Ki-67 data, the distinction between cases showing complete regression versus cases showing no regression (and omitting the cases with partial regression) on the basis of Ki-67 values (gained by any method) gives an area under the curve (AUC) of 0.969, whereas comparing complete regression values with the cases with partial regression and those showing no regression aggregated together, the AUC turns to 0.93 (Figure 3). Here the suggested best cut-off value to predict complete regression would have been around 56% LI (sensitivity: 0.89; specificity: 0.81).

Figure 4 gives an insight into the distribution of individual Ki-67 LIs behind the mean values shown in Method 3 related part of Figure 2. Although the mean values shown in Figure 2 are obviously higher (more to the right) for cases with pCR (red fields) than for the rest of the cases, it is clear that the individual cases show considerable overlaps. Although most red fields fall to the right and above 50%, there are a few blue and yellow fields (representing no regression and partial pathological response, respectively) falling among the red fields and a few red fields falling among the other colors and below 50%. Similar overlaps could have been represented for all methods investigated.

Discussion

Ki-67 immunohistochemistry is one of the most common methods to prognosticate biologic behavior of breast carcinomas. Precise evaluation of the Ki-67 LI is quite important for pathologists because this factor is also considered in therapeutic decision making, especially when chemotherapy is indicated in addition to hormonal treatment [4,9-15]. Although Ki-67 immunohistochemistry provides more or less obvious nuclear positivity in the cells within the cell cycle, including proliferating tumor cells, it is still a real challenge for pathologists to perform an objective evaluation. A precise and exact evaluation is complicated by numerous factors like tumor heterogeneity, intensity of staining to be considered positive, admixture of non-tumor cells with tumor cells, the intensity of counterstaining with hematoxylin and sometimes small, non-representative tumorous area, to list only a few of these factors. Beside these issues, another important factor is the time taken by the evaluation. Although an international consensus recommends the examination of at least 500, but optimally 1000 cells for deriving the Ki-67 LI [22], this practice is rarely followed. In everyday practice, pathologists are reluctant to spend the required time for counting hundreds of tumor cells on each case. It is also obvious from personal discussions, that many pathologists believe that counting the Ki-67 LI on the basis of 1000 cells is a waste of time. Our first study highlighted the limitations of daily practice based tumor cell counting, as all three participating pathologists counted only a hundred cells, and the effort resulted in poor reproducibility of the Ki-67 LI [23]. That study showed that the Ki-67 LI values were significantly influenced by the person of the investigator. Not only the inter-observer, but also the intra-observer agreement was found to be poor to moderate. A later study of ours suggested that by choosing a limited area of the tumor representing the highest proliferation of the core biopsy sample and fitting into a single digital image, and by helping to choose which cells to count with a grid, the reproducibility of the Ki-67 LI can be improved [24].

As mentioned previously, the Ki-67 LI has prognostic value in breast cancer and may indicate the need for additional chemotherapy in special cases. Cytotoxic therapy acts on proliferating cells, therefore it would be very logical to hypothesize that tumors with a higher baseline proliferation may better benefit from chemotherapy than those with a low baseline proliferation. In keeping with this theoretical approach, a better response to neoadjuvant chemotherapy was reported in tumors with high baseline Ki-67 LI [9, 26-28]. In contrast, tumors with a high Ki-67 LI showed similar response rate (77%) to chemoendocrine therapy than those with a low Ki-67 LI (81%) in a retrospective series from the Royal Marsden Hospital [29]. It is also likely that Ki-67 applied as a dynamic marker (i.e. taken as a baseline value and also during neoadjuvant therapy) may better predict the response and the outcome of disease [26, 27, 30], but multiple biopsies are not always easy to obtain. Our results displayed in Figure 2 show that breast cancers showing pCR consistently had a higher mean Ki-67 LI than those not responding or showing only partial pathological response whatever the method of evaluation or the antibody used were or whoever the observer was. Although this difference was seen among the small groups showing regression, looking into the details demonstrates that the regression of individual cases of breast carcinoma cannot be precisely predicted (Figure 4), and prediction on the basis of high versus low Ki-67 LI does not work for the individual cases. The only thing that can be stated is that tumors with higher Ki-67 LIs are more likely to regress completely, independently of the method of evaluation.

The methods compared in this study had different reproducibility [23, 24]. Although a good to excellent correlation was found between the results gained by different methods, antibodies and observers, the interobserver and intraobserver reproducibility of the everyday practice based counting on glass slides (Method 1) was only fair to moderate. Reproducibility of the Ki-67 LI has improved when the field to be assessed was limited to a digital image, chosen to represent the highest proliferation in the core biopsy sample (Method 2) [23, 24]. Interestingly, reproducibility was not worse when the proportion of stained cells was only estimated (Method 3) rather than counted (Methods 1 and 2). This is in keeping with the results of a very carefully designed study by Varga et al, which showed that the reproducibility achieved by estimation was not worse than that reached by meticulous counting [31]. This suggests that the simple approach of estimating by eyeballing has also diagnostic value, and may be better received by the pathological society as a simple and fast method, taking 4 to 12 times less time than counting [23, 24].

Although all of our study cases received neoadjuvant chemotherapy on the basis of locally advanced stage according to general practice and recommendations, biological markers, including those reflecting a high proliferation rate might be more suitable to select patients with potential benefit from this treatment. Pathological complete regression is associated with better prognosis [32, 33], and tumors achieving pCR have a higher mean Ki-67 LI than those which do not achieve pCR, in keeping with earlier works [9, 26-28]. However, proliferation alone is not sufficient to predict the response to neoadjuvant chemotherapy [28], as demonstrated by Figure 4, and it is also a matter of debate what cut-off value should separate tumors with high and low KI-67LIs. In this respect, Figure 2 seems useless, and suggests that depending on the methods, antibodies and observers, different cut-offs could be used for this purpose. Fasching et al used a >13% cut-off [9], in keeping with the value suggested for the immunohistochemistry based separation of luminal A and luminal B carcinomas [18, 34,]. A recent analysis by members of the European Working Group for Breast Screening Pathology found that the distribution pattern of 1709 Ki-67 LI values has peaks at values ending with 0 or 5, and therefore an inclusive or non-inclusive cut-off ending with these values would seem more realistic [unpublished results], and independently, the latest St Gallen recommendations have happily adopted a 20% cut-off in keeping with the cited results. Figure 2 would suggest that with most

of the methods included, a cut-off of 50% would delineate high proliferation, and the ROC curve analysis also supports a similar value (i.e. 55%) but the analysis of the distribution pattern by the members of the European Working Group for Breast Screening Pathology cited previously suggests that only a minority of breast cancers would fall into this category, as the median Ki-67 LI of the large cohort was 17% whereas the estrogen receptor-positive cases had a median of only 14% [unpublished data]. Therefore, the small subset of locally advanced breast cancers analyzed in the present work would fall into the higher end of the Ki-67 LI values of a general breast cancer cohort, and might not be representative enough. This would also counteract the determination of a cut-off value. Individual cases with high proliferation (e.g. Ki-67 LIs above 50%) would still have overlapping Ki-67 LI values with non-regressing tumors (Figure 4).

As to the best method, statistics do not allow to suggest that any one of these methods is better than the other, they are just different from each other with regard to the Ki-67 LIs. It seems obvious that Method 3, the estimation made on the digital image taken from the area thought to represent the highest proliferation in the core biopsy sample had substantial reproducibility [24], is fast and simple, and seems to separate responders from non-responders better than the other methods. However, individual cases would fail to follow the prediction even on the basis of this small sample. (The number of data evaluated in this study did not allow an ROC curve analysis of sufficient value to estimate the best cut-off value for this individual method.) Response to therapy is obviously a phenomenon depending on multiple factors of which the number of cells in the cycle is only one.

Conclusion

Tumors achieving pCR after neoadjuvant chemotherapy had a higher mean Ki-67 LI than those that achieved either partial pathological response or did not show regression. This statement is true for all the 3 antibodies tested, all the 4 observers and all the methods evaluated. Therefore, the simplest methods of evaluating the Ki-67 LI by eyeballing rather than time-consuming counting could be a good alternative to assess the proliferation rate and can be of use to predict response to primary systemic treatment, although the prediction based on the Ki-67 LI values alone will fail in some cases, representing the minority. The estimation of a cut-off value would require a larger series of cases.

Conflict of interest statement

The authors have no conflict of interest to declare.

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Figure 1

An example of the digital pictures analyzed. A: image used for estimating the Ki-67 LI by eyeballing (Method 3); B: the same image with parallel grid lines laid over delineates the stained and unstained cells (those touching the lines or crossed by them) to be considered when counting (Method 2).

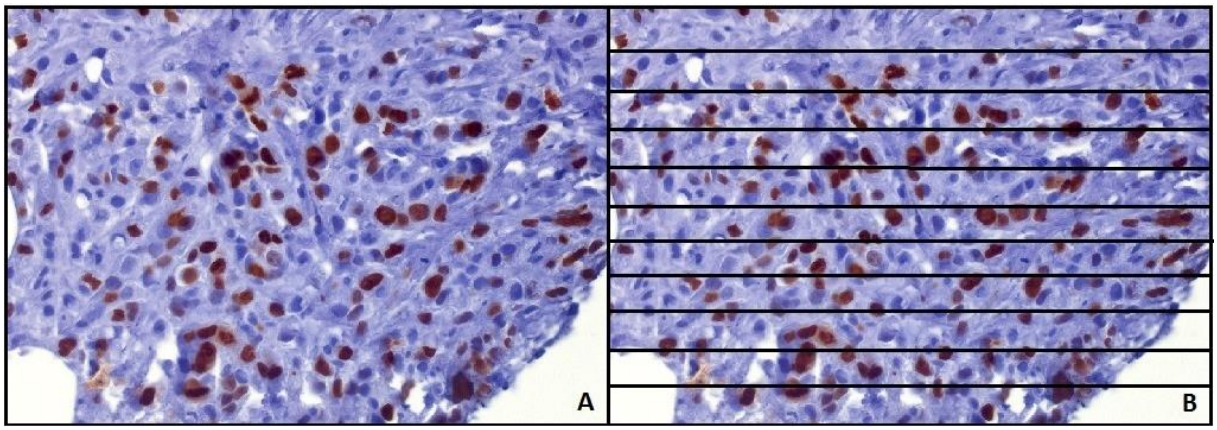


Figure 2

Mean Ki-67 LIs gained by four different evaluation methods by 4 observers and 3 antibodies in the different categories of tumor regression after neoadjuvant chemotherapy. Blue fields represent values of tumors with no regression, the yellow ones are for tumors with partial regression and the red ones for those with pathological complete regression. The green color reflects the fusion of blue and yellow for instances when the mean values of partial regression and no regression fall in the same field. Each colored field represents the mean KI-67 LI value of ten cases in one of the three groups (no regression, partial regression and complete regression). GC, AV, EC, BK reflect the observers and MIB1, SP6, B56 the antibodies. The scale of the figure is by 5%, i.e. each field represent a range of values with 5 integers from 1-5 to 96-100.

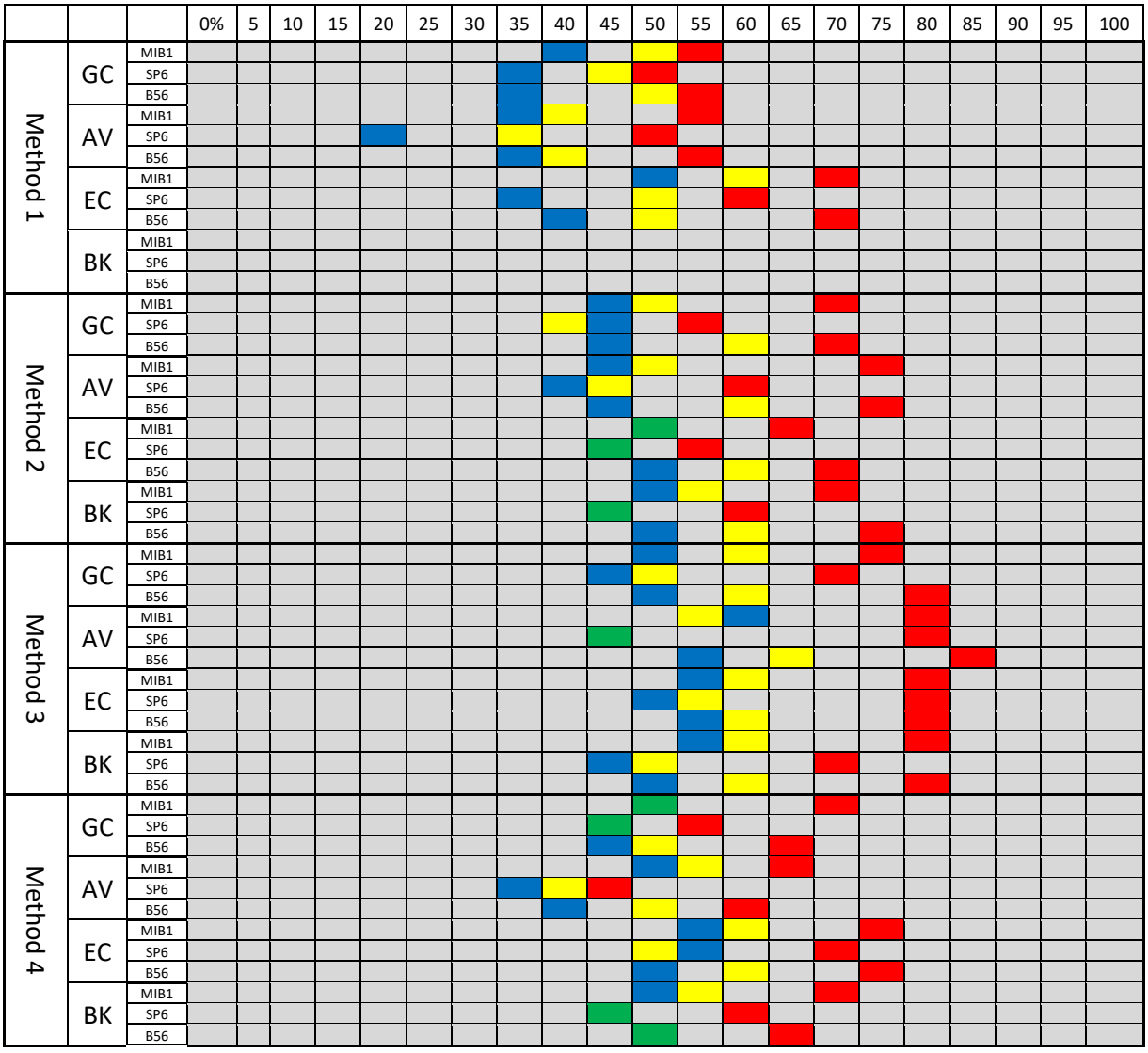
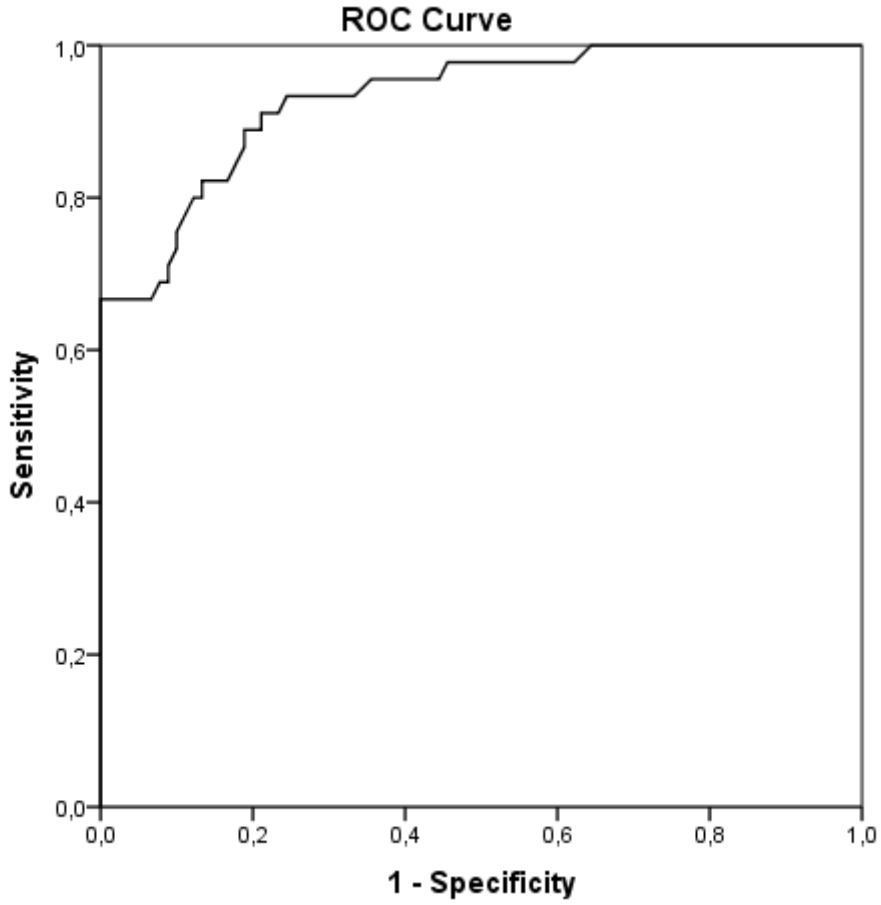


Figure 3

ROC curve derived from all average Ki-67 values (all methods, observers and antibodies) as predictors of the group with pCR and those without it (partial response and no response together)



AUC: 0.93 (sensitivity: 0.89; specificity: 0.81)

Figure 4

Individual Ki-67 LI values for Method 3 (estimation on representative digital images). Blue fields represent values of tumors with no regression, the yellow ones are for tumors with partial regression and the red ones for those with pathological complete regression. Each colored field represents a case with a given Ki-67 LI. GC, AV, EC, BK reflect the observers and MIB1, SP6, B56 the antibodies with 30 cases falling in each of the assessments. The scale of the figure is by 5%, as the estimation was done in steps of 5% precision.