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METHOD

A New Ki-67 / E-Cadherin Cocktail Reduces Inter-observer Variation of the Calculated Proliferative Index

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Abstract The proliferative index in breast carcinoma is usually calculated by the percentage of the Ki-67 positive cells out of the total number of malignant cells. In order to reduce the inter-observer variability of the calculated proliferative index a cocktail of antibodies against E-Cadherin and Ki-67 (Ki/Cad Cocktail) is presented. The cocktail was applied on 59 cases of infiltrating duct carcinoma of breast and compared to the consecutive slides stained for Ki-67 alone. The Ki/Cad cocktail has the advantage that by adding the anti E-Cadherin antibody, all the malignant epithelial cells are highlighted and can be differentiated from other proliferating cells. Statistical analysis proved that the cocktail increases the inter-observer agreement from 89 % to 97 % as compared to the Ki-67 alone and also reduces the overlap between the cancer grades.

Keywords Ki-67 · E-Cadherin · Cocktail immunostain · Proliferative index · Infiltrating duct carcinoma of breast

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Introduction

Breast cancer is the commonest cancer in women both in the developed and the developing world. The incidence of breast cancer is increasing in the developing world due to increased life expectancy, increased urbanization and adoption of the western lifestyle. Carcinoma of the breast accounts for nearly 30 % of cancers diagnosed in women in the United States, and it is the second leading cause of cancer death among women. Approximately 230,480 new cases of invasive breast cancer and 39,520 breast cancer deaths were expected to occur among US women in 2011. Breast cancer incidence rates were stable among all racial/ethnic groups from 2004 to 2008. A woman's risk of developing this disease increases with age [1].

Proliferative activity is regarded as an important prognostic indicator. Ki-67 is expressed by proliferating cells in late G1, S, G2 and M phases but not in resting cells G0. The most used antibody for its detection in formalin fixed tissues is a monoclonal antibody against MIB-1 (Ki-67). The percentage of Ki-67 positive cells out of the total number of malignant cells is defined as the proliferative index. Generally, elevated proliferative index correlates with a worse prognosis in cancer. In breast carcinoma the proliferative index is important for estimating the prognosis; it plays a role in the decision on treatment and also in monitoring the effect of chemotherapy. The Ki-67 index has been analyzed in various series of breast cancer, and was found to be of significant prognostic value [2–8]. The proliferative index is calculated as the percent of Ki-67 positive cells out of all the malignant cells; however, the variability between observers is quite high [9]. It is difficult to count all the malignant cells which are not proliferating, because we have to rely on their nuclear stain alone. The international cancer working group recommends optimizing the degree of counterstaining, given that Ki-67 negative nuclei determine the overall malignant population for calculating the proportion of Ki-67 positive cells. Weak counterstaining can

result in overestimating of Ki-67 index [10]. Another problem is to avoid considering proliferating, Ki-67 positive lymphocytes or soft tissue cells as breast cancer cells. Therefore it is important to highlight the malignant epithelial cells. In a previous report [11] we presented the Ki-67 & E-Cadherin Cocktail (Ki/Cad Cocktail) composed in our Department. E-cadherin stains the membrane of infiltrating duct carcinoma cells while Ki-67 stains the nuclei of the proliferating cells. It is possible to apply these 2 antibodies simultaneously against two compartmentally localized proteins i.e. the membranal E-cadherin and the nuclear Ki-67. It is easy to recognize all the malignant cells which are highlighted by E-Cadherin antibody and to avoid counting lymphocytes or stromal cells which are negative for E-Cadherin. The purpose of the present study was to examine 59 cases of infiltrating duct carcinoma, immunostained for Ki-67 alone as compared to the Ki/Cad Cocktail. The slides were examined by 3 experienced pathologists who calculated the proliferative index for each case. The intra-observer and inter-observer agreement were examined statistically for the Ki-67 group and the Ki/Cad Cocktail group.

Materials and Methods

Consecutive 59 lumpectomy specimens of infiltrating duct carcinoma, from our routine biopsies were examined. Formalin fixed paraffin embedded tissue blocks were cut at 5 microns thickness and stained with Hematoxylin & Eosin.

Immunohistochemistry

Representative paraffin blocks were chosen and immunostained with a cocktail consistent of equal amounts of two antibodies: monoclonal mouse anti human Ki-67 antigen Clone MIB1 at a concentration of 1:100 (Dako, Denmark) and Mouse anti-E cadherin (clone: 4A2C7 Zymed laboratories San Francisco California) at a 1:50 concentration, which we named Ki/Cad Cocktail. Consecutive sections from the corresponding paraffin blocks were stained also for the same clone of MIB1 alone at a 1:200 concentration. The staining was performed on the Ventana Bench mark, (Tucson, Arizona, USA) according to the manufacturer's instructions using iView DAB detection kit and mounted manually with Pertex. The sections were examined by three experienced pathologists using the guidelines how to calculate the proliferative index [10].

All observers were blinded to their own results and to the results of the other investigators.

Statistical Analysis

The inter-rater reliability testing was performed by use of the intra-class correlation- coefficient (ICC) [12, 13]. Kappa<0.4 represented poor agreement, 0.4–0.6 moderate agreement, 0.6–0.8 substantial agreement, > 0.8 almost perfect agreement. The intra-observer variability (for Ki-67 and Ki&Cad cocktail) was calculated for each observer by a paired two sample t-test for means.

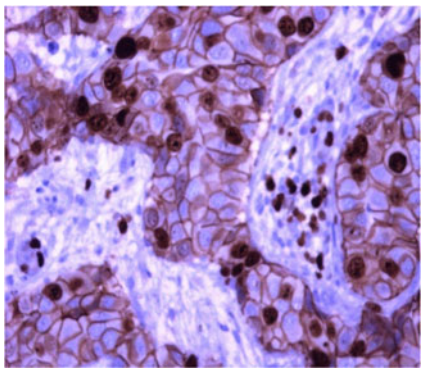

Fig. 1 Infiltrating duct carcinoma of breast, Ki/Cad cocktail immunostain × 20. Note the brown membrane E-Cadherin staining of the malignant epithelial cells and the nuclear staining of Ki-67 in some of the malignant cells. Note also the proliferating lymphocytes with no E-Cadherin membrane staining

Results

All the tumors were infiltrating duct carcinomas, grade 1–3. Ki-67 is a nuclear stain which stains any kind of proliferating cell. E-Cadherin stains the membranes of the infiltrating ductal, epithelial cells and does stain neither lobular epithelial cancer cells nor the lymphocytes. Due to these differences, it was easy to recognize the malignant epithelial cells and to calculate their proliferative index, without being biased by counting Ki-67 positive lymphocytes or omitting Ki-67 negative cancer cells. In Fig. 1 the double immunostain with the Ki/Cad cocktail is seen in an area of infiltrating duct carcinoma of the breast which shows a membranal staining of E-Cadherin in all the malignant epithelial cells and some of them show nuclear positivity for Ki-67. There are some Ki-67

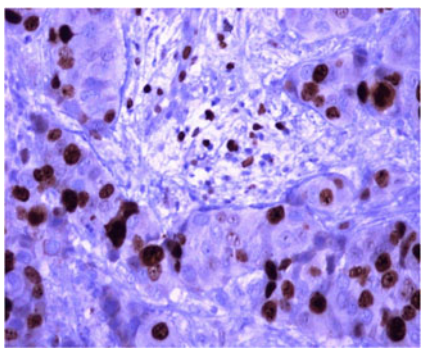

Fig. 2 Infiltrating duct carcinoma of the breast stained by Ki-67 only. The larger cells are the malignant cells, some of them are Ki-67 positive, but it is difficult to recognize all the non-proliferating malignant cells. There are also some proliferating lymphocytes

Table 1 The proliferative indices of all the cases according to each observer and the immunostain method used

Case#	Grade	Observer 1		Observer 2		Observer 3		Overall Kappa	
		Ki67	Ki/Cad	Ki67	Ki/Cad	Ki67	Ki/Cad	Ki67	Ki/Cad
1	1	2	1	2	1	2	3	0.89	0.97
2	1	7	2	7	2	5	3		
3	1	1	1	1	1	1	2		
4	1	2	1	2	1	5	2		
5	1	5	2	1	2	5	3		
6	1	5	5	5	3	5	3		
7	1	1	1	0	1	1	1		
8	1	2	2	1	1	1	1		
9	1	1	1	1	1	1	1		
10	1	2	1	3	2	2	1		
11	1	5	2	4	2	3	2		
12	1	3	2	4	1	1	1		
13	1	6	4	8	4	5	2		
14	1	7	3	6	2	6	3		
15	1	2	1	4	2	4	2		
16	1	13	4	12	4	8	5		
17	1	5	1	2	1	2	2		
18	1	1	1	2	1	1	1		
19	1	2	3	2	3	5	2		
20	2	20	10	20	14	30	10		
21	2	5	8	15	12	20	10		
22	2	80	5	15	5	30	8		
23	2	10	10	42	9	20	10		
24	2	70	8	30	12	20	10		
25	2	70	10	48	18	30	20		
26	2	30	10	30	25	20	15		
27	2	7	5	5	9	10	5		
28	2	30	10	43	20	30	10		
29	2	50	10	38	18	50	10		
30	2	40	20	35	20	50	10		
31	2	30	20	20	20	50	10		
32	2	20	10	10	7	20	10		
33	2	20	20	30	20	40	10		
34	2	75	15	55	20	62	15		
35	2	34	15	30	10	20	10		
36	2	20	10	28	12	20	10		
37	2	35	15	70	17	50	20		
38	2	50	15	50	20	40	15		
39	2	40	15	35	20	50	25		
40	3	40	30	60	40	40	50		
41	3	80	60	90	40	80	30		
42	3	70	30	70	25	80	50		
43	3	90	80	90	90	90	90		
44	3	80	70	50	70	50	70		
45	3	50	30	50	25	80	30		
46	3	40	40	40	40	60	50		
47	3	60	30	60	25	80	40		
48	3	90	70	80	70	90	70		

Table 1 (continued)

Case#	Grade	Observer 1		Observer 2		Observer 3		Overall Kappa	
		Ki67	Ki/Cad	Ki67	Ki/Cad	Ki67	Ki/Cad	Ki67	Ki/Cad
49	3	80	60	50	60	80	60		
50	3	70	25	50	25	40	25		
51	3	60	50	40	50	60	40		
52	3	90	80	90	80	90	80		
53	3	50	40	80	50	60	40		
54	3	60	30	80	25	80	30		
55	3	70	30	60	30	70	30		
56	3	90	90	90	90	90	90		
57	3	60	50	60	40	80	50		
58	3	60	70	60	60	90	70		
59	3	90	80	80	80	90	80		

positive cells with no E-Cadherin membrane staining. These are proliferating lymphatic cells.

Figure 2 shows the same case stained for Ki-67 alone. It is difficult to recognize all the non-proliferating malignant cells. Note also that there are some proliferating lymphocytes which could be mistaken for malignant cells.

Table 1 shows the proliferative indices as reported by the three expert pathologists. The results with the Ki/Cad cocktail were: grade 1 tumors had a mean index of 1.98, range 1–5. Grade 2 tumors had a mean index of 13.2, range 5–25 and grade 3 tumors had an index of 52.16, range 25–90. With anti-Ki-67 alone the indices were 3.54 (1–13), 34.11 (5–80) and 69.83 (40–90) for grade 1,2 and 3 tumors respectively. The distribution of the proliferative indices of the different tumor grades had less overlapping cases with the Ki/Cad cocktail than with the anti Ki-67 alone.

Statistical analysis showed that the inter-observer correlation coefficient for Ki-67 alone was 0.89 with 95 % confidence interval of 0.83–0.93, while that of the cocktail was 0.97 with 95%confidence interval of 0.96–0.98.

Intra-observer agreement: For each case all the observers reported a higher proliferative index by Ki-67 alone than by the Ki/Cad cocktail. The t-test for paired samples for mean was significant $P<0.001$.

Discussion

A cocktail of antibodies is a combination of antibodies blended together into one solution in order to apply on the tissue section at the same time. Leaders in the field of immunohistochemistry stated that "immunohistochemistry cocktails are here to stay", and that cocktails improve reproducibility among pathologists and the accuracy of diagnosis [14]. There are many types of antibody cocktails, for instance Pan Keratin antibody is a combination of antibodies against the different keratin types (high and low molecular weight) and thus will stain the cytoplasm of most epithelial cells. Another example is the Pan-melanoma antibody cocktail [15] which contains antibodies against HMB45, MART-1 and Tyrosinase. It showed high sensitivity to all forms of malignant melanoma. Another type of cocktail is a cocktail that stains different cell types of the tissue, for instance P504S/p63 double cocktail. The P-63 stains the prostatic basal cells of the normal glands and P504S stains the prostatic cancer cells. Together they enhance the diagnosis of prostatic carcinoma [16–18]. This cocktail is available commercially with double chromogen visualization. There are also 3-antibody cocktails [19]. P504S stains the cytoplasm of malignant cell with one chromogen while p63 and 34betaE12 stain the basal cells with another chromogen. There is also a commercially available CD20/Ki-67 multivision cocktail which by co-localization of the two antibodies may help to determine the proliferative index in B cell lymphoma. Our cocktail, which has an antibody with nuclear reactivity (Ki-67) and another antibody with cell membrane reactivity (E-Cadherin) is based on the same principle, but it is novel because there is no need for double color visualization and it can be prepared in every Pathology laboratory.

E-Cadherin is a calcium-regulated adhesion molecule expressed in most normal epithelial cells. Qureshi et al. [20] found that 99.5 % cases of infiltrating duct carcinoma were positive for E-cadherin. In our study all the epithelial cells were positive for E-cadherin, however E-cadherin is not expressed in the lymphocytes, soft tissue cells or lobular breast carcinoma; therefore our cocktail is particularly well suited to estimate the proliferative index in infiltrating duct carcinoma of the breast. The advantages of the Ki/Cad cocktail are that it highlights all the malignant cells; it facilitates the accurate estimation of the

proliferative index and promotes inter-observer agreement. Mengel et al. [9] showed mean concordance of 75.7 % between observers for the Ki-67 proliferative index. Our results show mean concordance of 89 % when Ki-67 was evaluated alone, but it rose to 96 % when the Ki/Cad cocktail was evaluated.

The cocktail is inexpensive as there is no need for the use of a double chromogen kit and it can be assembled in any routine immunopathology laboratory. In conclusion: The Ki/Cad cocktail offers a reliable and cost-effective way of evaluating the proliferative index in infiltrating duct carcinoma biopsies. Further investigations will show if it can be used for Fine needle aspiration of breast and/or for other epithelial tumors.

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