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ORIGINAL ARTICLE

Analysis of membranous Ki-67 staining in breast cancer and surrounding breast epithelium

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Abstract

Membranous Ki-67 staining with the MIB-1 antibody has been described in hyalinising trabecular adenomas of the thyroid and sclerosing haemangiomas of the lung. Its relatively rare occurrence in breast tumours has also been documented. The aim of the present study was to assess the rate of any membranous MIB-1 staining in breast specimens. The staining was performed at room temperature with 1:100 dilution of the antibody. One hundred four core needle biopsies and 41 operative specimens were analysed. Membranous staining was noted in 36/144 invasive carcinomas, 20/42 in situ carcinomas and 46/99 cases of peritumoural benign/normal breast epithelium. Most often, it presented as focal and partial polarised luminal membranous staining although complete circumferential staining also occurred, and membranous labelling was sometimes accompanied by cytoplasmic staining, too. In a few cases tested, greater dilution of the primary antibody did not abolish the membranous staining, which was absent with the SP6 monoclonal Ki-67 antibody. The membranous staining of invasive tumours showed no association with histological grade, lumen formation, oestrogen or progesterone receptor status or the Ki-67 nuclear labelling. In contrast, it was associated with a HER2-positive status, although it occurred in all molecular subtypes approached by immunohistochemistry. The background of this membranous staining remains elusive. It is unlikely to represent an artefact. At least partial sharing of an epitope of the nuclear Ki-67 protein with an unidentified membranous protein and some functional differences between membranous staining producing tumours and tumours lacking this pattern of staining may both contribute to some extent.

Keywords Breast cancer · Ki-67 · MIB-1 · Membranous staining · Immunohistochemistry · HER2

Introduction

Proliferation of malignant tumours is closely related to their progression and prognosis. Ki-67, as detected by immunohistochemistry (IHC), is widely used as a proliferation marker because it is expressed through the cell cycle, except the G0 phase [1]. Several studies have proven its prognostic value in breast cancer [2, 3], and this marker also made its way to influence therapeutic decisions [4]. The distinction between luminal A-like and luminal B-like breast cancers by the IHC-based surrogate classification is also influenced by the Ki-67 proliferation rate, although the cut-off value for the discrimination has changed time to time [5–7]. There are many issues which lack standardization in the context of Ki-67 IHC evaluation, and these include the antibodies used as well as the methods of assessment and quantification. Therefore, an international panel set up and made recommendations in order to harmonise the practice of Ki-67 evaluation in breast cancer [8]. These recommendations suggest that, optimally, the MIB-1 mouse monoclonal antibody should be used as this is the most studied and most widely applied one [8]. Despite these recommendations, national guidelines vary, and the reproducibility of the Ki-67 labelling index as a proliferation marker seems less than optimal to base treatment decisions on it [9]. In Hungary, Ki-67 immunostaining of breast carcinomas has become more and more commonly asked for by the oncological community, and this staining was included in a recent consensus recommendation on the histopathological work-up of breast cancers [10].

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Ki-67 staining is a nuclear one, of which the intensity varies during the cell cycle, being the highest in the M phase and much lower in G1 and S phases [3]. A membranous (and cytoplasmic) staining pattern has also been recognised as one of differential diagnostic values: this is seen in hyalinising trabecular tumour (HTT) of the thyroid gland but not in papillary thyroid carcinoma to which this HTT resembles [11]. Another tumour, which has been described to show membranous (and cytoplasmic) staining for Ki-67 as opposed to lung cancer, is pulmonary sclerosing haemangioma [12]. Although Leonardo et al. were unable to reproduce this staining pattern in their eight cases of pulmonary sclerosing haemangiomas, they observed the membranous staining on six HTTs, four invasive breast cancers and single cases of sarcomatoid mesothelioma and oncocytoma, respectively [13]. The reported immunolabelling was rather diffuse and presented circumferentially on the outer surface of the cells and was occasionally associated with cytoplasmic staining, too. These authors also demonstrated that the membranous staining pattern is typical for the MIB-1 clone (both from DAKOCytomation, Glostrup, Denmark, 1:300 dilution and Immunotech, Marseille, France, 1:300 dilution) at room temperature and is not seen with other antibodies (7B11, Zymed, San Francisco, 1:200 dilution; KIS5, DAKOCytomation, Glostrup, Denmark, 1:300 dilution; KI88, Biogenex, San Ramon, kit dilution) or at a higher temperature (37 °C) [13]. On the contrary, some authors have found identical staining intensity and ratio at room temperature and at 37 °C [14].

Although reported as early as 2007 [13], membranous staining of breast tumours is not widely recognised, and when encountered for the first time, it may give rise to the suspicion of a technical failure. In this study, membranous Ki-67 staining was systematically evaluated along with the nuclear staining for which the IHC reaction was done, and was noted in a surprisingly high proportion of cases.

Materials and methods

According to the International Ki-67 in Breast Cancer Working Group recommendations [8], no tissue microarrays were used; the immunostainings for Ki-67 were performed on core needle biopsy (CNB) specimens or operative specimens, in 2017. All tissues were fixed in buffered formalin and embedded in paraffin. During the period involved, two different batches of the MIB-1 monoclonal antibody (Dako, Glostrup, Denmark, Catalogue number: M7240) were used in sequence, at a dilution of 1:100 (incubation at room temperature for 1 h) after 45 min of heat-induced epitope retrieval at 98 °C in pH 6 citrate buffer. The author assessed Ki-67 nuclear labelling by estimation on the whole tumour area seen on the slide [15] and rounding off the values [16], therefore reducing the values to a limited possibilities (i.e. 1, 2, 3, 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80 or 90%). Additionally, as part of the routine work-up, the oestrogen receptor (6F11, Leica Biosystems, Novocastra, Newcastle, UK), progesterone receptor (Pgr312, Leica Biosystems, Novocastra, Newcastle, UK) and human epidermal growth factor receptor-2 (HER2) statuses (4B5, Roche-Ventana, Tucson, AZ, USA) were also determined. The evaluation of these stainings followed the Hungarian guidelines [10], which themselves follow the American Society of Clinical Oncology [17] and United Kingdom guidelines [18] for the relevant testings. Specimens with a 2+ IHC score for HER2 were tested by chromogenic in situ hybridisation (Zytodot 2C SPEC ERBB2/CEN 17 kit, ZytoVision, Bremerhaven, Germany).

Additionally, a pair of cases were stained with the MIB-1 antibody at dilutions of 1:200 and 1:400 and with the SP6 (Histopathology Ltd., Pécs, Hungary) and MM1 (Biocare, Concord, CA, USA) antibodies, too.

Ki-67 membranous staining was screened at medium power (× 100), and the slides were scrutinised for any membranous staining in invasive and in situ carcinomas as well as normal breast epithelium or benign lesion when present on the slide used for the evaluation. The policy of interpreting any membranous staining as positive was chosen in parallel with the interpretation of nuclear Ki-67 labelling, where any intensity is taken as positive and reflecting the cell being in the cell cycle [8]. One slide was used in each case, and this generally included either three 1-mm-large cores for biopsy samples or a full-face cut-surface from a standard tissue block for operative specimens.

Statistical analyses were performed with VassarStats (available at http://vassarstats.net; Richard Lowry, Vassar College, Poughkeepsie, NY; accessed 05–10 January 2018). The 95% confidence interval (CI) of proportions was calculated with a correction for continuity. Categorical variables were analysed for an association with membranous Ki-67 staining with the chi-square test (according to Yates, with correction for continuity); when the number of elements was too low, the Fisher exact test was used instead, with two-sided p values < 0.05 considered significant.

No ethical approval was deemed necessary for this non-interventional study requiring no patient identification or data, using only slides which were available as part of the routine work-up.

Results

Altogether, 145 specimens of 144 patients were assessed; one patient had two tumours sampled by CNB; 135 tumours were primary carcinomas, nine were recurrences and one tumour tested was an adenomyoepithelioma. The material investigated consisted of 104 CNB samples and 41 resection samples. There were no differences between the proportion of either

invasive or in situ carcinomas or normal/benign epithelium demonstrating membranous staining in the two types of specimen.

Membranous staining was identified in 36 invasive carcinomas. It was most often luminal ($n = 20$), sometimes circumferential over the luminal surface even if not complete ($n = 9$) or demonstrated the combination of these two patterns ($n = 7$). Some cancers showed luminal ("membranous") labelling in intracytoplasmic vacuoles too, and cytoplasmic staining also occurred in a minority of the tumours (Fig. 1).

The relation of any membranous Ki-67 staining and different aspects of the carcinomas investigated is shown in Table 1. No association was found with pT and pN categories (as components of the anatomical stage [19]). Lobular carcinomas showed this pattern much less commonly (1/19; 5.3%, 95% CI 0.3–28.1%) than ductal (no special type, NST) and tubular carcinomas (35/123, 28.5%; 95% CI 20.9–37.4%) (Table 1), and the difference in staining between lobular and non-lobular carcinomas was also significant ($p = 0.044$, Fisher's exact test). The lobular carcinoma demonstrating a membranous staining showed this feature focally around the intracellular vacuoles. The single cases of tubulolobular, mucinous, invasive micropapillary and invasive solid papillary carcinomas showed only nuclear Ki-67 positivity, although one NST tumour, with less than 50% micropapillary component, showed the characteristic inside-out polarised membranous staining typical of this histological type, which is generally seen with the MUC1 and some other antibodies [20] (Fig. 1b). The observation that most of the membranous staining was luminal suggested that lumen formation might be a feature influencing the phenomenon of membranous labelling. However, the highest proportion of membranous positivity occurred with a tubule formation grading score of 2, and neither the score for tubule formation nor the histological grade was associated with membranous Ki-67 staining. There was also no association between membranous Ki-67 staining and ER or PR status and the Ki-67 (nuclear) labelling index (either with a cut-off of 15% or with one of 20%), but a significant association was found with the HER2 status and molecular subtypes approached by an IHC-based surrogate classification. HER2-positive tumours showed the highest labelling, but membranous staining was seen in all types of carcinomas (Table 1).

19/40 (47.5%, 95% CI 31.8–63.6%) DCIS, 1/2 (50.0%, 95% CI 2.7–97.3%) LCIS and 46/99 (46.5%, 95% CI 36.5–56.7%) associated benign lesions or normal breast tissue showed membranous labelling with Ki-67. In 10 DCIS and 33 cases of normal/benign epithelial structures, these membranous positivities were seen without membranous staining of the invasive tumour. Less than half (71/145, 49.0%; 95% CI 40.6–57.4%) of the assessed slides were completely devoid of membranous staining. Forty-two slides included both in situ and invasive carcinoma, and membranous staining was seen in both components in 11 (26.2%; 95% CI 14.4–42.3%). Thirty-one of these slides also included at least some normal breast parenchyma or benign lesions, and membranous Ki-67 positivity was seen in all three components in 4 (12.9%; 95% CI 4.2–30.8%).

Discussion

After the first description of four cases of breast cancer demonstrating membranous MIB-1 labelling at room temperature by Leonardo et al. [13], a larger series was published including 322 breast carcinomas, of which 26 (8%) showed membranous (and cytoplasmic) staining with the Dako antibody at a dilution of 1:50 at both room temperature and 37 °C [14]. Four of these cases showed an apical/luminal type staining, whereas 22 were reported to have a cytoplasmic positivity with accentuation on the cytoplasmic membrane. Although diffuse and strong membranous positivity was more common, focal and weak positivity also occurred. As no cut-off staining proportion or intensity was mentioned, it is most likely that any staining was considered positive, similarly to the present study. The presence of membranous staining was correlated with histological grade 3, an ER-negative and a HER2-positive status, although membranous staining failed to be an independent marker of poor prognosis in a multivariate model [14]. Another study with 27/136 (20%) membranous/cytoplasmic staining rate of either primary breast and/or metastatic nodal carcinomas also found a similar association with the ER status [21].

In contrast to the tissue microarray-based study summarised above [14], membranous MIB-1 staining occurred in the majority of the cases reported in the present article. It was not limited to invasive carcinomas but DCIS and normal breast epithelium also showed this feature; the staining of these latter two components did generally not follow the staining of the invasive carcinoma on the same slide. The membranous staining was generally focal, sometimes weak (Fig. 1h) or limited to a few cells (Fig. 1d) and needed meticulous search for identification, but in other cases, it was diffuse and strong, noticeable without search. Another discrepancy from the previously cited study [14] was the more commonly seen polarised luminal staining rather than complete circumferential staining of the cells. This pattern is reminiscent of the focal to diffuse CD10 membrane positivity seen in apocrine lesions [22]. An area of micropapillary differentiation in an NST carcinoma (but not in the single micropapillary carcinoma case) also demonstrated the inside-out pattern of polarised membranous staining which is typical of this histological type of cancer with the MUC1 antibody [20]. The much higher rate of positivity reported in this series may be related to the commonly focal nature of Ki-67 membranous staining, which

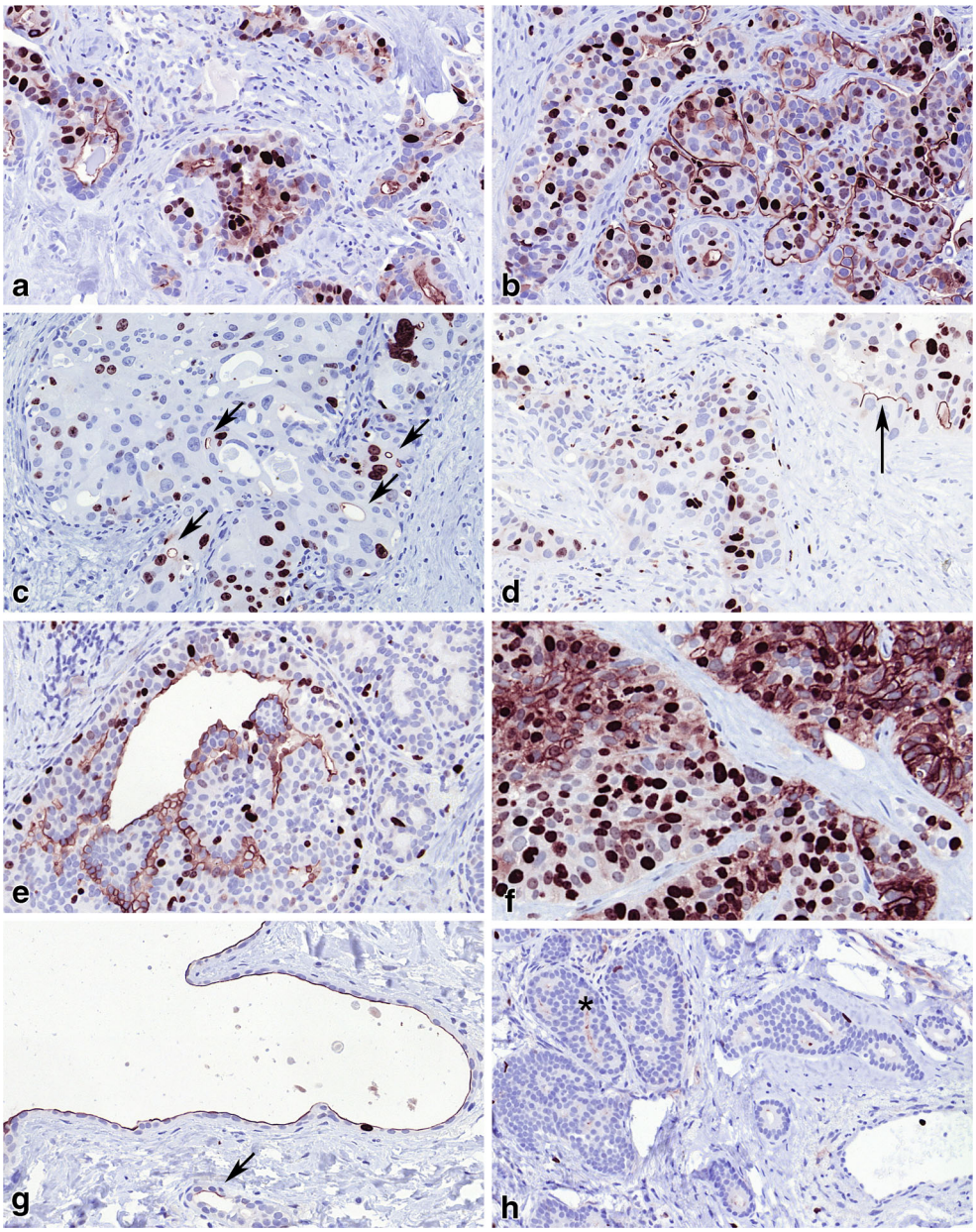

Fig. 1 Examples of MIB-1 staining patterns seen in parallel with the expected nuclear staining. **a** Polarised (luminal) membranous staining of an NST breast cancer demonstrating minor areas of invasive micropapillary carcinomas shown in part **b**. **b** Micropapillary area of the tumour shown in part **a**, demonstrating a typical inside-out pattern. **c**: Focal intracytoplasmic vacuole "membranous" staining (arrows). **d** Minimal luminal membranous staining (arrow) requiring specific search for detection. **e** DCIS demonstrating luminal membranous staining, but probably due to the plane of sections, some cells also show a circumferential membranous labelling. **f** A tumour with focal circumferential membranous staining of some cells with or without nuclear and/or cytoplasmic staining. **g** Benign cyst with circumferential luminal staining and smaller gland with focal luminal membranous staining (arrow). **h** Minimal weak focal luminal staining of a cribriform low grade DCIS (asterisk) (**a**, **b**, **c**, **d** and **h** ×40; **e**, **f** and **g** ×70)

may therefore be underestimated in tissue microarrays. The method used here, scrutinizing the slides for even very focal staining, can also have some role in the discrepancy.

The origin of such a membranous staining with an antibody detecting a nuclear antigen remains unresolved. Three possible explanations have been proposed. First, an artefactual nature was formulated, but all authors agree that the pattern of staining with its crisp character and features like the limitation of the staining to HTT and not to papillary carcinomas in the thyroid is so much against an artefact and that the two other possibilities are much likelier. Either a cross-reacting or homologous epitope [11–13] or a functional translocation of the antigen or its part [14] may play a role in it.

Ki-67 is a large protein coded by MKI67 mapping to 10q26.2. The National Institutes of Health protein database [23] contains two *Homo sapiens*-derived isoforms of the proliferation marker protein Ki-67, which are 3256 and 2896 amino acids (AAs) long, respectively. These two proteins share the 2896 AAs of the shorter isoform 2, but there are 360 consecutive AAs in addition in the primary structure of the protein "inserted" at AA position 136 in the longer isoform 1; i.e. isoform 2 lacks an in-frame exon in the 5′ coding region as compared to isoform 1. It is unknown whether the isoforms react differently with any of the antibodies (or especially the MIB-1 antibody) used for the IHC detection of Ki-67 and whether their existence can contribute to the membranous localization of the reported staining. The fact that only MIB-

Table 1 The relation of membranous Ki67 staining and histological parameters of the tumours

	Membranous Ki67+	Membranous Ki67−	
pT categories			$p = 0.896$
Tx	11	38	
T1mi, T1a, T1b	3	12	
T1c	10	31	
T2	10	22	
T3, T4	2	5	
pN categories			$p = 0.337$
Nx	13	42	
N0	13	45	
N1	9	14	
N2, N3	1	7	
Histological type			$p = 0.071$*
NST (ductal)	33	85	
ILC	1	18	
Other	2	5	
Or			$p = 0.044$*
NST (ductal) + tubular	35	88	
ILC	1	18	
Histological grade			$p = 0.383$
1	10	21	
2	11	46	
3	14	39	
Score for tubules			$p = 0.056$
1	4	8	
2	15	26	
3	16	73	
ER status			$p = 0.108$
Positive	27	95	
Negative	9	13	
PR status			$p = 0.052$
Positive	23	88	
Negative	13	20	
HER2 status			$p = 0.007$
Positive	11	11	
Negative	25	97	
Ki67			$p = 0.566$
High (> 15%)	21	55	
Low (≤ 15%)	15	53	
Or			$p = 0.624$
High (> 20%)	16	41	
Low (≤ 20%)	20	67	
IHC surrogate subtypes			$p = 0.030$ (df = 4); $p = 0.015$ (df = 3)
TN	5	10	
HER2 + (ER−)	4	3	
HER2 + (ER+)	7	8	
LUM B-like (> 15%)	7	37	
LUM A-like (− 15%)	13	50	
Or			$p = 0.021$ (df = 4); $p = 0.010$ (df = 3)
TN	5	10	

Table 1 (continued)

	Membranous Ki67+	Membranous Ki67−	
HER2 + (ER−)	4	3	
HER2 + (ER+)	7	8	
LUM B-like (> 20%)	3	24	
LUM A-like (− 20%)	17	63	
DCIS			$p = 0.999$
LG	3	3	
IG	7	7	
HG	8	10	

df degree of freedom (4 with all categories together; 3 with HER2 positives taken as a single category), *NST* no special type, *ILC* invasive lobular carcinoma, *TN* triple-negative, *HER2+* Human epidermal growth factor receptor 2-positive, *ER+* oestrogen receptor-positive, *LUM A* luminal A, *LUM B* luminal B, *DCIS* ductal carcinoma in situ, *LG* low grade, *IG* intermediate grade, *HG* high grade

*All *p* values are from chi-square test, except those labelled with an asterisk, where the Fisher exact test (two sided) was used

1 gives the membranous reaction would be logically related to a specific cross-reaction of the antigen recognizing Fab part of this antibody (and not the others) with a membranous component and the nuclear Ki-67 protein. The temperature-related staining differences seem more difficult to explain, but may be related to the alterations of the tertiary structure of the antigen detected on the membrane. The nuclear and membranous staining showed no association (Table 1), and there were cells with either both or none of these staining patterns. The focal nature of the membranous staining could suggest a spurious reaction, but the fact that, often, the focal staining was polarised to the luminal surface of the cell membrane and an association with HER2 overexpression (see below) would be against a false reaction. The scarcity of the related literature and the data at hands do not allow a clear statement about the origins of the membranous MIB staining in different tissues, and the possible explanations remain hypothetical; the present author prefers the cross-reaction of epitopes theory.

When five antibodies were compared, the membranous staining was seen only with the MIB-1 antibodies from two different sources, and three other antibodies failed to give this staining in addition to the nuclear one [13]. In an independent study, MIB-1 was compared with a fourth monoclonal antibody, BGX-Ki67 (Biogenex, Fremont, CA, USA; 1:75 dilution), and again, membranous staining was only seen with MIB-1 [21]. The membranous/cytoplasmic staining with MIB-1 did not vanish with any of three different antigen retrieval methods, two different detection systems and in either of the two laboratories doing the staining [21]. Although the aim of the present study was only the assessment of the prevalence of MIB-1 membranous staining in breast lesions and normal structures, a pair of cases were evaluated with alternative stainings. The original dilution of the MIB-1 antibody (1:100) was within the range proposed by the manufacturer (1:75–1:150), but further dilutions (1:200 and 1:400) were also used, and although there was a slight reduction in membranous (and nuclear) staining, further dilution of the primary antibody did not abolish the reaction. Two different batches of MIB-1 were used during the period invested, and membranous staining occurred with both. There was no membranous staining with the SP6 rabbit monoclonal Ki-67 antibody (Fig. 2) and the MM1 mouse monoclonal antibody (not shown). These results are in keeping with previous reports [11–13]. Similarly to differences in nuclear labelling [24], the antibodies are different in membranous labelling, too; MIB-1 appears to be the only antibody where the membranous (/cytoplasmic) pattern has been described. The differences in staining and their characteristics listed above are very likely to be related to the differences in the epitopes recognised by the different antibodies, although the exact epitopes are not known. The above data point to a specific MIB-1-antibody-antigen reaction shared between the nuclear Ki-67 protein and an unknown membranous epitope, which may or may not be part of the Ki-67 molecule. Differences in qualitative (yes or no) nuclear labelling are considered to reflect whether the cell is in the cell cycle or not, whereas differences in quantitative labelling (intensity of staining) are interpreted as differences in different phases of the cell cycle. On the other hand, there are many technical and probably biological issues which may also influence the nuclear staining, which is antibody dependent and demonstrates differences according to the antibody used [24].

Similar qualitative and quantitative differences in membranous (/cytoplasmic) MIB-1 staining are more difficult to explain and remain a mystery. Although the membranous Ki-67 staining has been related to “a peculiar neoplastic phenotype” [13], because the staining was mainly recognised in benign and malignant tumours, the present results suggest that even normal breast epithelium may display this pattern of Ki-67 labelling. Rare “luminal border” of normal thyroid follicles and occasional alveolar side surface membrane Ki-67 immunopositivity were noted by the early descriptions of

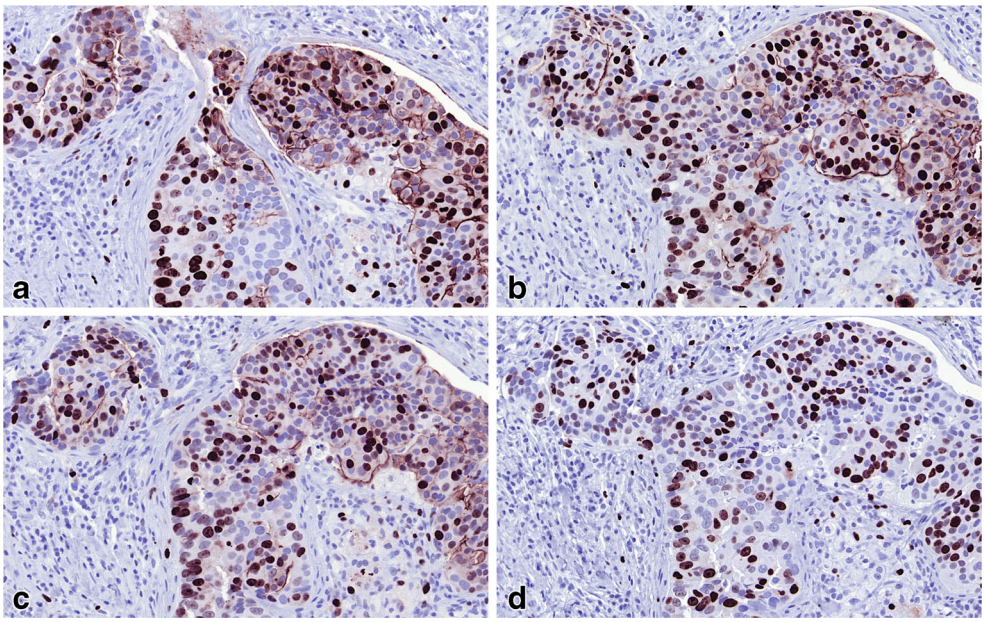

Fig. 2 Alternative Ki67 stainings tested in the study. Same area of a tumour with MIB-1 membranous staining at dilutions 1:100 (**a**), 1:200 (**b**) and 1:400 (**c**) lacking this staining pattern with the SP6 antibody (**d**) (**a**–**d** ×40)

membranous Ki-67 staining in HTT [11] and pulmonary sclerosing haemangiomas [12], respectively. While collecting the material for this analysis, a bronchial biopsy demonstrating focal luminal MIB-1 staining of bronchial glands and epithelium was also identified (Fig. 3), reinforcing that the membranous staining pattern is not restricted to neoplastic lesions, and staining of the normal epithelium is not restricted to breast tissues.

Only a few specimens demonstrated membranous staining in all three compartments (normal/benign, in situ carcinoma and invasive carcinoma), and matching positivity of the DCIS and invasive component was only seen in about a quarter of the cases. In a study documenting 27/136 cases with membranous MIB-1 staining in either the primary or the nodal metastatic breast carcinoma, about a half of the cases ($n = 14$) had membranous staining at both sites [21]. The lack of a complete match of membranous staining between in situ carcinoma, invasive carcinoma and metastatic carcinoma may be related to the focal nature of the membranous staining and to the fact that less than the whole of the tumour was investigated. However, it can also reflect that the membranous staining has nothing to do with a "genuine" feature or function of the neoplastic (or normal) tissues. Contrasting with this last possibility, some functional differences have been noted between tumours with and without membranous MIB-1 labelling.

An association between an ER-negative status and membranous MIB-1 labelling was reported in two independent studies [14, 21]. Although there seemed to be a higher proportion of ER-negative carcinomas that demonstrated membranous staining in this series, too (41 vs 22%), there was no statistical difference in the rate of membranous staining of ER-negative and ER-positive tumours (Table 1). In contrast, HER2 positivity was associated with the membranous staining, similarly to previous reports [14, 25]. The molecular subtypes approached by IHC were also associated with the membranous staining pattern, probably because of the combined influences of the HER2 and ER statuses. In our study, the MIB-1 nuclear labelling index showed no association with membranous staining, and this was the case in a previous

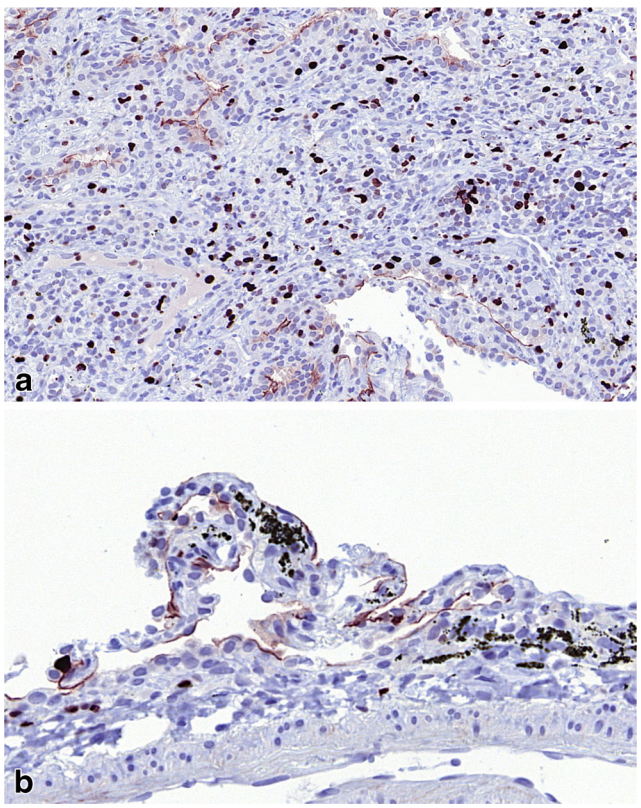

Fig. 3 Bronchial biopsy with luminal membranous staining of bronchial glands and epithelium (**a** ×40; **b** ×70)

study, too [14], but others have found a higher Ki-67 nuclear labelling with an alternative antibody in cases with membranous MIB-1 staining (BGX-Ki67) [21]. Interestingly, three cases were found to have a membranous/cytoplasmic MIB-1 labelling without nuclear staining but a high nuclear labelling with the alternative Ki-67 antibody used in parallel [21]. These data together would suggest the contribution of a biological factor as the explanation for the membranous MIB-1 labelling, which, however, would be difficult to consider behind the membranous staining of normal breast epithelium.

Finally, it may be mentioned that diffuse and continuous membranous staining has been observed as an artefact with the SP6 antibody in a multiplexed immunofluorescent system, but this was clearly due to the non-specific binding of the SP6 anti-Ki-67 antibody to the secondary antibody detecting HER2 on the membranes, and could be specifically blocked [26]. This artefactual membranous staining has therefore nothing to do with the MIB-1-related membranous staining, but highlights a potential confounding factor that can step in while trying to clarify the origin of the membranous MIB-1 labelling.

In summary, a relatively high proportion of breast specimens have been reported to demonstrate at least focal membranous labelling with the MIB-1 Ki-67 antibody. This staining pattern, along with nuclear labelling, was seen in both normal/benign epithelium, in situ and invasive carcinomas. Although membranous staining in carcinomas was associated with HER2 positivity, it is difficult to explain why some tumours show such membranous staining and others do not and why some areas in a tumour show a membranous staining while others do not.

Author contribution The author of the manuscript made substantial contributions to the following:
- the conception/design of the work; the acquisition, analysis, interpretation of data for the work;
- drafting the work and/or revising it critically for important intellectual content;
- final approval of the version submitted for publication; and
- agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.
Other contribution is acknowledged in the Funding section of the manuscript.

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Compliance with ethical standards

The author has consulted the journal policy regarding compliance with ethical standards and state that accepted principles of ethical and professional conduct have been followed. The author includes information regarding sources of funding and potential conflicts of interest (financial or non-financial) (next section). Ethical approval and informed consent-related information (waiver for this particular study) are summarised in the final paragraph of the "Materials and methods" section. The study did not include animals; therefore, issues relating to animal welfare do not apply.

Conflict of interest The author declares that he has no conflict of interest.

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