TECHNICAL REPORT

Comparison of Ki-67 equivalent antibodies

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Aims: To compare commercially available Ki-67 equivalent antibodies with regard to qualitative and quantitative immunohistochemical staining characteristics.

Methods: The following antibodies were used: monoclonal MIB-1 (Immunotech), monoclonal MM1 (Novocastra), polyclonal NCL-Ki-67p (Novocastra), and polyclonal Rah Ki-67 (Dako). All immunostainings were evaluated in squamous epithelium from formalin fixed and paraffin wax embedded pharyngeal tonsils. Labelling indices (LIs) were recorded twice to test their reproducibility.

Results: By application of all four antibodies the nuclear staining could be either diffuse, granular, or a combination of both (classified as granular in this study). The diffuse pattern generally showed a strong or moderate staining intensity, whereas the granular pattern displayed a continuum from strong to very weak, making it difficult to discriminate between positive and negative nuclei. The diffuse staining pattern was seen in approximately 59% of the nuclei with the MIB-1 antibody and in 35–45% when the other antibodies were used. The following mean LIs were recorded: MIB-1, 31%; NCL-Ki-67p, 21%; Rah Ki-67, 17%; and MM1, 14%. The reproducibility was excellent for all four antibodies, with the mean of differences between the two runs of counts ranging from 1.1% to 1.5%.

Conclusions: The four tested Ki-67 equivalent antibodies revealed differences in qualitative and quantitative staining characteristics, which resulted in considerable variations in registered LIs. The MIB-1 antibody appears to have a higher sensitivity for detecting the Ki-67 antigen than the other three tested antibodies. These differences are important to consider when proliferative activity is determined by the Ki-67 LI.

Counting of mitoses is the classic method used to determine proliferative activity in normal and neoplastic tissues by light microscopy. However, this method registers only the M phase of the cell cycle and the number of identifiable mitoses also depends upon the period of time between surgical removal and fixation of the specimen.[1 2] Furthermore, strict morphological criteria for mitoses have to be used to avoid confusion with nuclear pyknosis and karyorrhexis.[3–6] Lack of such standard criteria, in addition to heterogeneous distribution of mitoses,[4 7] may explain the often poor reproducibility of mitotic counts.[3 8 9] Therefore, there has been a need for alternative methods to determine proliferative activity. However, several of the methods that have been developed have never been used in routine surgical pathology because they are not practical or are time consuming; examples of such methods are the determination of nucleolar organiser regions and 5-bromodeoxyuridine and tritiated thymidine labelling.

An important landmark was reached in 1983 when Gerdes *et al* reported the monoclonal antibody Ki-67, which recognises a proliferation specific nuclear antigen expressed during the G1, S, G2, and M phases, but not in the G0 phase.[10] Determination of the Ki-67 labelling index (LI) soon became widely used in surgical pathology. In most types of cancer (for example, carcinomas, sarcomas, lymphomas, and gliomas), the Ki-67 LI was found to correlate with tumour grade and clinical course.[11–14] However, a major drawback of this prototypic Ki-67 antibody is that it can be used on frozen sections only. Therefore, the discovery of the proliferating cell nuclear antigen (PCNA), which could be detected in paraffin wax sections after microwave heating, was met with much enthusiasm. However, it was soon realised that PCNA is not at all proliferation specific,[15–18] and many studies revealed a poor correlation between this antigen and other proliferation markers, in addition to clinical parameters.[19–22] Consequently, PCNA staining is no longer recommended for use in surgical pathology.[20 23 24]

During the past decade, several monoclonal and polyclonal antibodies against peptides from recombinant fragments of the Ki-67 antigen have been produced.[25–29] The epitopes detected by these Ki-67 equivalent antibodies resist formalin fixation and the immunostainings can thus be performed on paraffin wax embedded sections after antigen retrieval. The most frequently used antibody of this kind is MIB-1, which was first reported by Cattoretti *et al* in 1992.[25] The proliferative activity determined by MIB-1 has generally confirmed the findings obtained with the original Ki-67 antibody; that is, correlation with tumour grade,[30–32] clinical course,[33–36] and other methods for the evaluation of growth rate.[30 31 37]

> "A major drawback of the prototypic Ki-67 antibody is that it can be used on frozen sections only"

It is expected that the application of the various Ki-67 equivalent antibodies should produce similar staining results, although to our knowledge comparisons of the qualitative and quantitative staining properties of Ki-67 equivalent antibodies under standardised conditions have not been reported previously. Thus, our present study was undertaken to determine and compare the staining properties of four commercially available Ki-67 equivalent antibodies with special emphasis on the LIs.
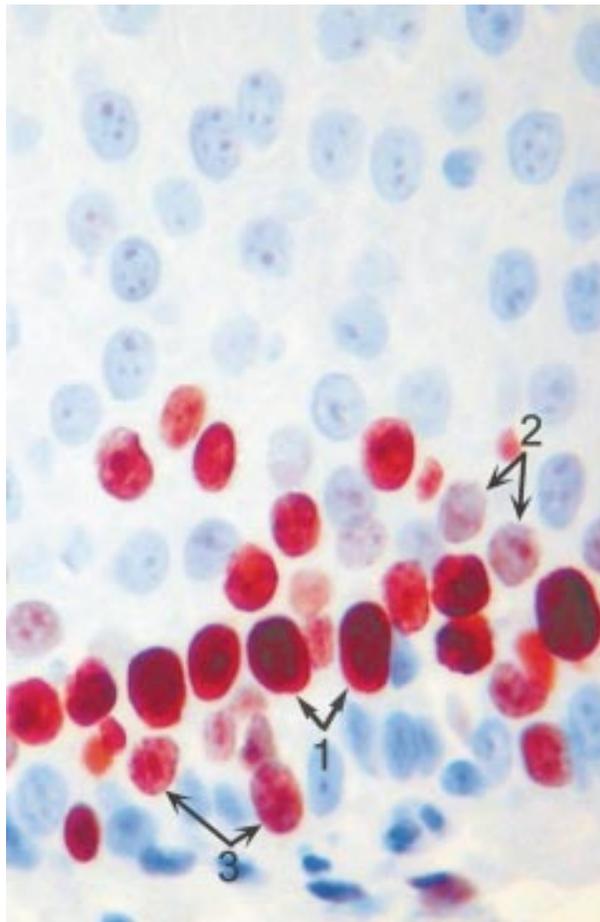
MATERIALS AND METHODS

We used pharyngeal tonsils obtained at tonsillectomy from 10 patients aged 32–63 years (four men and six women). Clinical indications for surgery were tonsillar hyperplasia, inflammation, or suspicion of malignancy. However, we only included in our study those tonsils without malignancy or inflammation involving the surface squamous epithelium. The specimens were immediately fixed in 4% phosphate buffered formaldehyde. Eight tonsils were fixed for approximately 24 hours, one for five days, and one for seven days before further processing and paraffin wax embedding.

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Table 1 Survey of the Ki-67 equivalent antibodies used in our study

Antibody	Clonality	Dilution	Manufacturer
MIB-1	Monoclonal	1/100	Immunotech, Marseille, France
NCL-Ki-67-MM1 (MM1)	Monoclonal	1/100	Novocastra Laboratories, Newcastle upon Tyne, UK
NCL-Ki-67p	Polyclonal	1/100	Novocastra
Rabbit antihuman Ki-67 antigen (Rah Ki-67)	Polyclonal	1/150	Dako AS, Glostrup, Denmark

For immunohistochemical stainings, 4 µm sections were incubated with the four Ki-67 equivalent antibodies listed in table 1 after microwave antigen retrieval. For information concerning antibody production details the reader is referred to the data sheets handed out by the suppliers. The optimal working dilution of each antibody was determined by titrations. An automatic immunohistostainer (TechMate 500; Dako, Glostrup, Denmark) was used with a standard avidin–biotinylated immunoperoxidase technique. The lymphoid tissue of the tonsils was used as an internal positive control in each case. In the negative controls the primary antibodies were omitted. All four antibodies produced an immune staining that should be regarded as specific for the Ki-67 antigen, with staining of nuclei at known proliferative locations, such as germinal centres and the basal half of the squamous epithelium, and with no background staining. In serial sections stained with the four antibodies, corresponding areas of the squamous epithelium were identified and marked with ink. Only areas with intact epithelium were evaluated and areas with leucocyte infiltration and prominent stromal papillae were omitted.

All sections were evaluated with regard to the distribution and intensity of the immunohistochemical reaction product. The following types of staining pattern were seen (fig 1): a diffuse staining of the nucleoplasm and a granular pattern, which stained nucleoli or granules of different size dispersed throughout the nucleoplasm. Some nuclei showed a mixed pattern; that is, strongly stained granules against a diffusely positive background of a lower staining intensity. We classified these nuclei as granular. The staining intensity of the diffuse and granular patterns was classified as strong, moderate, or weak.

The Ki-67 LIs were determined by light microscopy at ×400 magnification using an ocular grid. Two observers performed the counts independently of each other from the same areas of the epithelium. First, at least 1000 nuclei were counted throughout the whole thickness of the epithelium, and then all positively stained nuclei within the same area were counted. The LI was defined as the percentage of immunoreactive nuclei. The first observer counted all positive nuclei regardless of staining quality (granular and diffuse), whereas the second observer registered diffusely stained nuclei only. Both observers performed their counts twice from exactly the same area without any delay, keeping the ocular grid in place.

The statistical analysis of the data was done using the SPSS for Windows software (SPSS, Chicago, Illinois, USA). Spearman's rank correlation was used to assess the correlation between the different Ki-67 antibodies. Comparisons of the repeated countings and of the LIs were performed with one way and two way analysis of variance tests. p Values less than 0.05 were regarded as significant.

RESULTS

The intensities of the diffuse nuclear staining pattern were generally classified as strong or moderate, making it easy to discriminate between positive and negative nuclei. The granular pattern, on the other hand, displayed a continuum from a strong to a very weak staining intensity, often making it difficult to discern between weakly positive and negative nuclei.

The relative proportions of nuclei with granular and diffuse staining patterns can be calculated from the figures in table 2. Thus, by staining with the MIB-1 antibody, more than half of the nuclei (mean, 59%) showed a diffuse staining pattern, whereas this pattern was seen in only 35–45% of the nuclei when the other three stains were used.

Table 2 shows the Ki-67 LIs (both granular and diffuse staining patterns) obtained by staining with the four antibodies. As can be seen, there are considerable differences in the mean LIs, with the highest figure for MIB-1 (~ 31%), followed by NCL-Ki-67p (~ 21%), Rah Ki-67 (~ 17%), and MM1 (~ 14%). The MIB-1 LIs were significantly higher than those obtained with the other three antibodies, as was the case for NCL-Ki-67p compared with MM1. The correlation between the LIs of the four antibodies was variable. However, MM1 correlated positively with all the other antibodies and MIB-1 with Rah Ki-67 and MM1. There were no significant differences in LIs between the first and second counting.

Figure 1 MIB-1 stain of squamous epithelium displaying three nuclear staining patterns: 1, diffuse; 2, granular; and 3, combined diffuse and granular (avidin–biotin–peroxidase complex method).

Table 2 Ki-67 labelling indices in tonsillar squamous epithelium from 10 cases stained with four different Ki-67 equivalent antibodies

Case no.	First observer: granular and diffuse staining pattern												Second observer: diffuse staining pattern only											
	MIB-1			MM1			NCL-Ki-67p			Rah Ki-67			MIB-1			MM1			NCL-Ki-67p			Rah Ki-67		
	1st	2nd	Diff	1st	2nd	Diff	1st	2nd	Diff	1st	2nd	Diff	1st	2nd	Diff	1st	2nd	Diff	1st	2nd	Diff	1st	2nd	Diff
1	28.9	30.7	1.8	12.0	11.1	0.9	18.0	18.5	0.5	15.4	15.2	0.2	19.6	19.2	0.4	4.3	4.4	0.1	6.5	6.8	0.3	6.9	7.2	0.3
2	37.5	39.0	1.5	16.8	16.5	0.3	17.0	15.9	1.1	18.4	16.7	1.7	22.5	22.5	0.0	5.6	5.2	0.4	4.9	5.3	0.4	11.2	11.2	0.0
3	34.8	36.1	1.3	16.9	15.3	1.6	19.8	19.8	0.0	26.1	23.8	2.3	21.0	22.1	1.1	8.5	8.3	0.2	10.9	11.3	0.4	7.9	8.6	0.7
4	38.6	38.2	0.4	18.0	19.4	1.4	29.1	26.6	2.5	22.4	22.1	0.3	23.7	24.9	1.2	6.4	6.7	0.3	9.4	9.9	0.5	9.4	9.9	0.5
5	27.7	26.1	1.6	12.5	10.5	2.0	26.9	27.3	0.4	16.5	14.3	2.2	16.8	15.3	1.5	4.8	5.1	0.3	8.7	8.6	0.1	8.8	8.8	0.0
6	26.5	29.6	3.1	14.9	16.5	1.6	26.7	22.7	4.0	15.7	16.3	0.6	17.6	16.1	1.5	10.2	10.9	0.7	8.7	8.4	0.3	9.9	9.7	0.2
7	23.4	22.9	0.5	9.4	9.8	0.4	11.7	11.5	0.2	20.3	17.4	2.9	9.7	11.3	1.6	4.0	3.8	0.2	4.6	4.7	0.1	5.8	5.8	0.0
8	33.5	30.7	2.8	15.6	18.1	2.5	23.4	23.4	0.0	20.3	17.4	2.9	17.7	17.7	0.0	7.0	6.6	0.4	5.9	5.7	0.2	7.5	8.0	0.5
9	35.5	35.6	0.1	13.3	13.3	0.0	24.3	22.9	1.4	16.0	15.4	0.6	18.5	18.3	0.2	3.1	2.8	0.3	6.7	6.7	0.0	6.1	6.4	0.3
10	22.0	20.1	1.9	9.1	9.9	0.8	14.7	13.6	1.1	7.7	7.6	0.1	14.6	15.8	1.2	4.3	4.1	0.2	5.3	5.2	0.1	3.5	2.8	0.7
Mean	30.8	30.9	1.5	13.9	14.0	1.2	21.2	20.2	1.1	17.9	16.6	1.4	18.2	18.3	0.9	5.8	5.8	0.3	7.2	7.3	0.2	7.7	7.8	0.3

Diff, difference between the 1st and 2nd readings.

DISCUSSION

Determination of the proliferative activity by the use of the Ki-67 LI depends on several factors, the most obvious being interobserver variations in cut off values between weakly positive and unstained nuclei. Before commencing our present study, we met to agree upon qualitative and quantitative staining criteria. Furthermore, we have previously cooperated on immunohistochemical studies of proliferative activity[38 39] and therefore feel confident that our evaluations are in agreement with one another. In addition, several factors related to tissue handling and histotechnical procedures are important.[39 40] The aim of our present study was to evaluate the importance of the choice of primary antibody by comparing qualitative and quantitative staining characteristics of four commercially available Ki-67 equivalent antibodies. It might be expected that these antibodies would have a similar sensitivity and specificity, but their recognition of different epitopes of the Ki-67 antigen makes this questionable.

The nuclear distribution of the immunohistochemical staining product was found to be granular, diffuse, or a combination of both. This finding is in agreement with previous reports in which the staining sites have been accurately determined at an ultrastructural level and which have also demonstrated variations of the granular and diffuse staining patterns in the various phases of proliferation.[24 41–44] We found that the diffuse staining pattern was generally strongly or moderately intense, whereas the granular pattern could show the whole spectrum of intensities. Staining with the MIB-1 antibody revealed a predominance of diffusely stained nuclei (mean, 59%), in contrast to staining with the other three antibodies, in which this staining pattern was seen in 35–45% of the nuclei. Therefore, it is possible that the various Ki-67 equivalent antibodies recognise epitopes that are expressed differently throughout the various phases of proliferation. However, the relative proportions of the two staining patterns had no influence on the reproducibility of the LIs.

> "The large differences in Ki-67 labelling indices obtained by staining with various antibodies necessitate the exact identification of the antibody used in every case"

The most important finding of our present study is undoubtedly the considerable differences in Ki-67 LIs obtained with the four antibodies. Thus, the MIB-1 antibody stained more than twice as many nuclei as the MM1 antibody (means, 31% and 14%, respectively). The NCL-Ki-67p (mean, 21%) and Rah Ki-67 (mean, 17%) antibodies produced intermediate results, although they were closer to the MM1 values than to those of MIB-1. This indicates that the MIB-1 antibody has a higher sensitivity than the other three antibodies, and it also gives the best visual staining, with more diffusely and strongly stained nuclei. However, as mentioned above, this qualitative staining property does not improve the reproducibility of the LI, which is equally good for all four examined antibodies.

There have been many reports of correlations between Ki-67 equivalent antibodies and other proliferation markers. Of particular interest in this context is the demonstration of considerably higher LIs by staining with the MIB-1 and Rah Ki-67 antibodies compared with the prototypic Ki-67 antibody.[38 45 46] One explanation for this finding could be better preservation of epitopes after formaldehyde fixation and antigen retrieval than by freezing of the tissue. Rose *et al* found a qualitatively similar immune staining using MIB-1 and polyclonal Ki-67 antibodies, but quantitative comparisons were not performed.[23] To our knowledge, the only previous study that has presented relevant quantitative data is the study of Torp in 1997.[47] He determined Ki-67 LIs in 11 glioblastomas by staining with the prototypic Ki-67 antibody in addition to the same four Ki-67 equivalent antibodies that were used in our

Take home messages

- The MIB-1 antibody had a higher sensitivity than the other three antibodies, and also gave the best visual staining, with more diffusely and strongly stained nuclei, although the reproducibility of the labelling index (LI) was equally good for all four examined antibodies
- Obviously, these differences are important to consider when proliferative activity is determined by the Ki-67 LI, and the exact antibody used should be identified in all cases studied

study. The reproducibility was tested by performing two runs of counts for two of the antibodies (MIB-1 and Rah Ki-67). Torp found the following mean LIs: prototypic Ki-67, 5.7%; MIB-1, 6.1/9.4%; MM1, 7.9%; NCL-Ki-67p, 5.9%; and Rah Ki-67, 10.9/5.2%. Thus, Torp's study did not reveal higher LIs using the MIB-1 antibody, and the differences between the two runs of counts were larger than those between each of the antibodies. However, it should be emphasised that Torp counted at random in a neoplasm with known heterogeneity, whereas we counted systematically in constant areas. Therefore, the findings of these two studies cannot be compared directly.

The large differences in Ki-67 LIs obtained by staining with various antibodies necessitate the exact identification of the antibody used in every case. Based on our study, MIB-1 seems to have a higher sensitivity for the Ki-67 antigen than the other three tested antibodies. However, our findings should be confirmed by others and extended to various tissues. Obviously, our findings have serious implications when the Ki-67 LI is used as a criterion for tumour grading or for clinical prognostic indication.

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