

Technical report

The enhanced peroxidase one step method increases sensitivity for detection of Ki-67 in pituitary tumours

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Abstract

Aim—To compare the Ki-67 labelling index determined using the enhanced peroxidase one step (EPOS) method with that using the standard ABC technique in pituitary tumours.

Methods—Adjacent sections were immunostained using the EPOS and ABC techniques with the same Ki-67 antibody and same antigen retrieval method.

Results—The labelling index measurements with the EPOS Ki-67 antibody were significantly higher than when using the traditional ABC method, and there was a positive correlation between the two techniques when performed on the same cases. This suggests that a higher proportion of cells are within the cell cycle than previously thought, although it gives no direct information on the rate of proliferation of the tumour.

Conclusions—It appears that the EPOS system is not only more convenient but may be more sensitive than traditional techniques for detecting Ki-67 in the nuclei of cells, thus demonstrating more accurately which cells have entered the cell cycle.

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Keywords: pituitary tumour; cell proliferation; Ki-67

Differences in tumour behaviour may determine both the prognosis and the effectiveness of treatment, whether it be surgery, drugs, or radiotherapy. The prediction of tumour behaviour and response to treatment has led to interest in the assessment of the proliferative activity of tumours.¹ Immunohistochemistry using antibodies to Ki-67 and proliferating cell nuclear antigen (PCNA), which are expressed in cells that have entered the cell cycle, can be used to assess the proportion of the cells from a tumour that are within the cell cycle. The Ki-67 antibody was originally raised against a nuclear fraction of Reed-Sternberg cells and recognises an antigen which is present in the gap 1 (G1), synthesis (S), gap 2 (G2), and mitosis (M) phases of the cell cycle, but not the quiescent G0 phase.^{2,3} Until a few years ago the

Ki-67 antibody could only be used on fresh or frozen tissues, but the monoclonal antibody MIB-1 has now been developed against part of the Ki-67 molecule,⁴ and this can be used in formalin fixed, paraffin embedded sections prepared using antigen retrieval.⁵ More recently, enhanced peroxidase one step (EPOS) Ki-67 antibodies have been developed.⁶ The EPOS antibody consists of molecules of antibody and horseradish peroxidase bound covalently to dextran, and was developed by Bisgaard and colleagues as a sensitive immunohistochemical method.⁷

There are some potential practical problems associated with using Ki-67 to assess proliferation. The labelling index (percentage of positively stained nuclei) is often found to be variable between different fields within the same tumour, because heterogeneous distribution of proliferating cells can introduce the potential for sampling error.⁸ The values obtained for the Ki-67 index may vary between laboratories depending on storage and handling techniques, thus limiting the use of a direct comparison of Ki-67 labelling index values. Finally, it is important to note that the proliferation rate of tissue depends not only on the number of cells in the cell cycle but also on the time taken to complete the cycle, and on whether cells undergo programmed cell death. Since the Ki-67 labelling index only measures the former and gives no indication of the latter, a tumour could be proliferating rapidly and have a low labelling index, or be proliferating slowly but remain in G1 and so have a high labelling index. Despite these potential drawbacks, the assessment of proliferation using the labelling index does appear to provide valuable

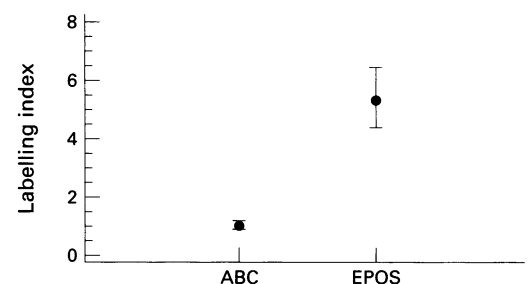


Figure 1 Labelling index using the ABC and EPOS techniques. Mean values and SEM.

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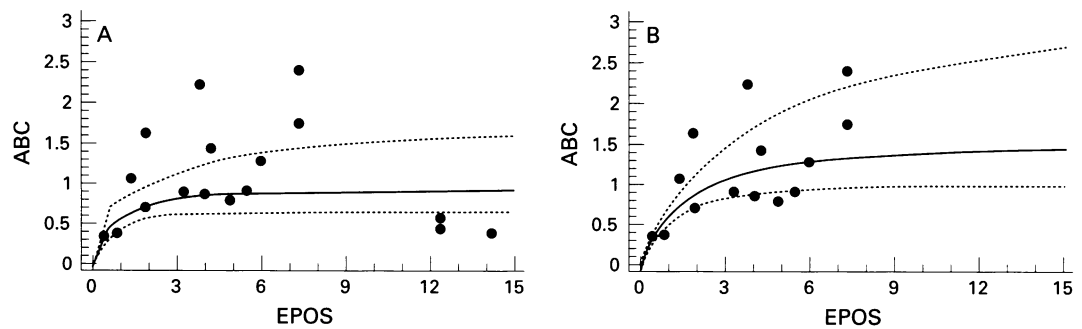


Figure 2 (A) Correlation between the labelling indices using ABC and EPOS techniques. (B) Correlation between the labelling indices using ABC and EPOS techniques excluding the three outliers. Continuous lines represent the best fitting regression curve (double reciprocal: $y = 1/[a + b/x]$); dotted lines represent 95% confidence intervals.

prognostic information in many tumour types, for example lymphomas, gliomas, and breast tumours.¹

We recently studied over 200 cases of pituitary tumour using the EPOS antibody for Ki-67 and showed higher labelling indices than other studies of pituitary tumours using the traditional ABC technique. Our aim in this study was to use the same rabbit antihuman Ki-67 antibody to compare the labelling index determined using the EPOS method with that using the standard ABC technique.

Methods

Seventeen surgically removed pituitary adenomas were investigated. The tissue was fixed in 4% buffered formalin, dehydrated, and embedded in paraffin. Sections 4 μ m thick were mounted on aptes (3-aminopropyl triethoxy silane, Sigma) coated slides, dewaxed, and rehydrated. The streptavidin-biotin peroxidase complex technique was used. An enhanced peroxidase one step (EPOS, Dako) rabbit antihuman Ki-67 antibody was applied as supplied (ready to use with no dilution required). Adjacent sections were immunostained using the standard ABC technique with the same rabbit antihuman polyclonal antibody to Ki-67 (Dako) used at 1:50 dilution.

Endogenous peroxidase activity was blocked using 3% hydrogen peroxide. Identical pre-treatment with microwaving in sodium citrate buffer, pH 6, was used for both types of antibody. Non-specific primary antibody binding was blocked using fetal calf serum at a dilution of 1:20 for the ABC Ki-67. The primary antibodies were all applied for 60 minutes at room temperature, and washed in buffered saline. For the ABC cases, an antirabbit biotinylated secondary antibody (Insight) was applied at 1:200 dilution for 30 minutes at room temperature, followed by washes and then by application of the horseradish peroxidase streptavidin complex (Dako) at 1:400 dilution for 30 minutes. Colour development was with metal enhanced diaminobenzidine (DAB) (Pierce and Warriner) applied for 15 minutes. The slides were lightly counterstained with haematoxylin.

Quantification of the Ki-67 labelled cells was performed using an image analysis system (VIDAS 21). Tumour cell nuclear staining was recorded as the percentage of positive cells

(labelling index). The labelling index was measured by a single observer without knowledge of tumour type. Statistical analysis (analysis of variance and non-linear regression analysis) was performed using the Statgraphics software package. A probability (*p*) value of < 0.05 was chosen to represent statistical significance.

Results

The labelling index (mean 5.3) with the EPOS Ki-67 antibody were significantly higher than with the traditional ABC method (mean 1.04) ($p < 0.05$) (fig 1), but there was a positive correlation between the two techniques when performed on the same cases ($R = 0.54$, $p = 0.02$) (fig 2A). There was no difference in distribution of positively labelled nuclei between the different methods, but the intensity of staining was higher with the ABC technique.

There were three outliers where the EPOS antibody produced a markedly higher labelling index than the ABC technique. These were all samples from invasive macroprolactinomas which tend to be the most aggressive form of benign pituitary tumour. When these were excluded from the analysis, the correlation improved to $R = 0.84$ (fig 2B).

Discussion

Although both the EPOS and standard ABC techniques are based on the same antigen retrieval techniques and the same antibody, the EPOS antibody demonstrated the presence of Ki-67 in the nuclei of significantly more cells than the ABC technique. This suggests that a higher proportion of cells are within the cell cycle than previously thought, although it gives no direct information on the rate of proliferation of the tumour. This is particularly relevant in the pituitary gland as alterations in cell cycle control involving the cyclins and related enzymes are currently being implicated in pituitary tumour pathogenesis.^{9, 10}

It is likely that, since the three outlying values were from specimens of invasive tumours, the EPOS antibody is detecting very small amounts of Ki-67 present in the cells in early G1 which the standard ABC technique does not detect. The EPOS antibody may thus be identifying those tumours that are potentially aggressive and need closer monitoring.

It appears therefore that the EPOS system may be more sensitive than traditional techniques for detecting Ki-67 in the nuclei of cells and so demonstrate more accurately which cells have entered the cell cycle. This illustrates the importance of stating the method of detecting Ki-67, and also that a particular technique should be used consistently if valid comparisons between specimens are to be made.

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