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# Heterogeneity of immunostaining for tumour markers in non-small cell lung carcinoma

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**Abstract**

Lung carcinoma is a leading cause of death. However, there are few indicators that can aid in prediction and prognosis. Many tumour markers are available, but their reliability is questionable. For example, Ki-67 expression has been associated with increased as well as decreased survival or with no clinical significance. The varying results have been attributed to the methodology, relative intensity of staining, variety of marking and statistical methods. To determine whether differential expression of markers within tumours may be a contributory factor to this lack of agreement, we used two marking methods to evaluate the level of expression of Ki-67, p53 and bcl-2, in addition to the apoptotic index, in serial sections of non-small cell carcinoma. All stains exhibited a degree of heterogeneity. This small study highlights the importance of standardisation of marking methods and interpretation of results if tumour markers are to be used as predictive or prognostic factors.


*Keywords:* Lung; Carcinoma; Immunohistochemistry; Ki-67; p53; bc1-2; Prognostic significance

## 1. Introduction

Lung carcinoma is a leading cause of cancer death in Europe and the United States of America (USA). However, there are few prognostic indicators available for clinicians on which to base their selection of a treatment regimen or to predict patient survival.

Tumour markers or oncogenes such as Ki-67, p53 and bcl-2, are the subject of ongoing research to try and to identify reliable predictive and prognostic indicators. The significance of these markers in relationship to disease progression and outcome remains unclear, as existing scientific and clinical studies are often associated with contradictory results.

Ki-67 is a protein associated exclusively with the active phases of the cell cycle (G1, S, G2 and mitosis) and is widely accepted as an accurate measure of proliferation. A high level of Ki-67 expression has been shown to be an indicator of increased survival [1–3], decreased survival [4,5] and as having no clinical significance [6–8] in non-small cell lung carcinoma. A similar disparity in results can be seen for mutant forms of the tumour suppressor gene *p53* [9,10] and *bcl-2*, a gene that prolongs cell survival by inhibiting apoptosis [11–13]. The level of apoptosis (apoptotic index) within a tumour has also been widely studied, again with conflicting results as to its significance [3,14,15].

The principal method for detecting these genes is using immunohistochemistry. The relative intensity of the resulting stain has been offered as an explanation for the variability in significance of tumour markers [16]; different intensities being associated with differing prognoses for the same gene product. The variety of marking methods used and their largely subjective nature [17] as well as the statistical analysis, inclusion criteria and risk factors [18,19] have also been suggested as possible explanations for these conflicting findings.

In order to determine whether differential expression of markers within tumours may be a contributory factor for the lack of agreement in previous studies, we looked at the level of expression of Ki-67, p53 and bcl-2, as well as the apoptotic index in serial sections of nine non-small cell lung carcinomas.

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## 2. Materials and methods

9 patients who underwent surgical resection for proven non-small cell lung carcinoma were randomly selected from histopathology records. A formalin-fixed paraffin-embedded block containing a high proportion of tumour was selected for each patient. Each block was cut into 4–6 μm thick sections, 10 serial sections for each antibody tested and five for determination of the apoptotic index. All blocks used were fixed in 10% neutral buffered formalin according to a standard histopathological protocol. All incubation times for the following experiments were ±1 min, unless otherwise stated, to ensure standardisation between the experiments.

### 2.1. Immunohistochemistry

The level of Ki67, p53 and bcl-2 expression in each specimen was determined using a horse radish peroxidase (HRP)-based commercial kit from Dako: EnVision™+. The sections were heated for 3 min at 60 °C until the paraffin started to melt then placed in three changes of Xylene, two changes of absolute and two changes of 95% ethanol (5 min each) to deparaffinise and rehydrate the tissue. Antigen retrieval was performed in a Sonia 10 CUFt 600 W microwave oven with 10 mM sodium citrate buffer. The buffer was heated for 5 min on full power before the slides were added then heated for a further 3 min (medium power), allowed to stand for 1 min then heated for a final 3 min (medium power). The slides/buffer was allowed to cool to room temperature before continuing. The EnVision kit was used according to the manufacturer's instructions. Briefly, the endogenous peroxide activity was blocked; primary antibody added (30 min) followed by the HRP-conjugated secondary antibody (30 min). A diaminobenzidine (DAB) chromogen was used to visualise the antigen (5 min). The slides were counterstained with haematoxylin, dehydrated and mounted in DPX medium. The Ki-67 antibody (mouse monoclonal IgG1 clone 556, Pharmingen) was used at 1/100 dilution, bcl-2 (mouse monoclonal IgG1, Dako) at 1/200 and p53 (mouse monoclonal IgG2b, Dako) at 1/400. Formalin-fixed paraffin embedded tonsil was used as a positive control for Ki-67 and bcl-2, while a breast carcinoma block was used for p53. A non-specific isotype-matched antibody was used as a negative control.

### 2.2. Apoptotic index

The apoptotic index was determined using the ApopTag$^{R}$ peroxidase plus kit from Intergen according to the manufacturer's instructions. Involuting mouse mammary gland was used as a positive control.

### 2.3. Analysis

The amount of staining in each slide was determined using two well recognised systems: a semi-quantitative method, the staining index, and a qualitative method, the Quickscore [20]. The staining index was determined by randomly selecting an area that showed positive staining, then counting 1000 nuclei and expressing the proportion of positive cells as a percentage. The index was calculated for two random positively staining areas of each slide. The Quickscore method involved grading the entire tissue specimen on two criteria, A (intensity) and B (quantity), then multiplying the result to obtain a score. This takes into account the proportion of cells positively stained and the relative intensity of the staining. The slides were analysed by two independent markers to minimise the subjectivity associated with the marking methods employed. Variation in the staining and marking of the slides was analysed using the coefficient of variation formula, coefficient of variation (CV%) = Standard Deviation (S.D.)/Mean ×100.

## 3. Results

### 3.1. Correlation between methods of assessment

There was good correlation between the staining index and the Quickscore method of assessing for Ki-67 ($R^2 = 0.62$), p53 ($R^2 = 0.80$) and bcl-2 ($R^2 = 0.87$) immunoreactivity (Fig. 1(a)–(c)). However, there was poor correlation ($R^2 = 0.022$) between these methods for ApopTag staining (Fig. 1d). Overall, a strong agreement was seen between the scores assigned by the two independent observers. This showed that the marking systems used are reliable and reproducible methods of assessing immunoreactivity of these tumour markers.

### 3.2. Intertumoral variation

The staining index for the nine tumours (calculated as the mean of the score obtained for nine separately stained consecutive sections from each tumour) ranged from <1 to 25% for Ki-67 (median 9%), 0 to 75% for p53 (median 29%), 8 to 43% for bcl-2 (median 31%) and 1 to 4% (median 1%) for ApopTag. No significant correlation was seen between the level of expression of any of the markers (data not shown).

### 3.3. Intratumoral variation

The staining index for each of the nine tumours (mean score of nine consecutive sections ±S.D.) for the four markers studied is shown in Fig. 2(a)–(d). The coefficient

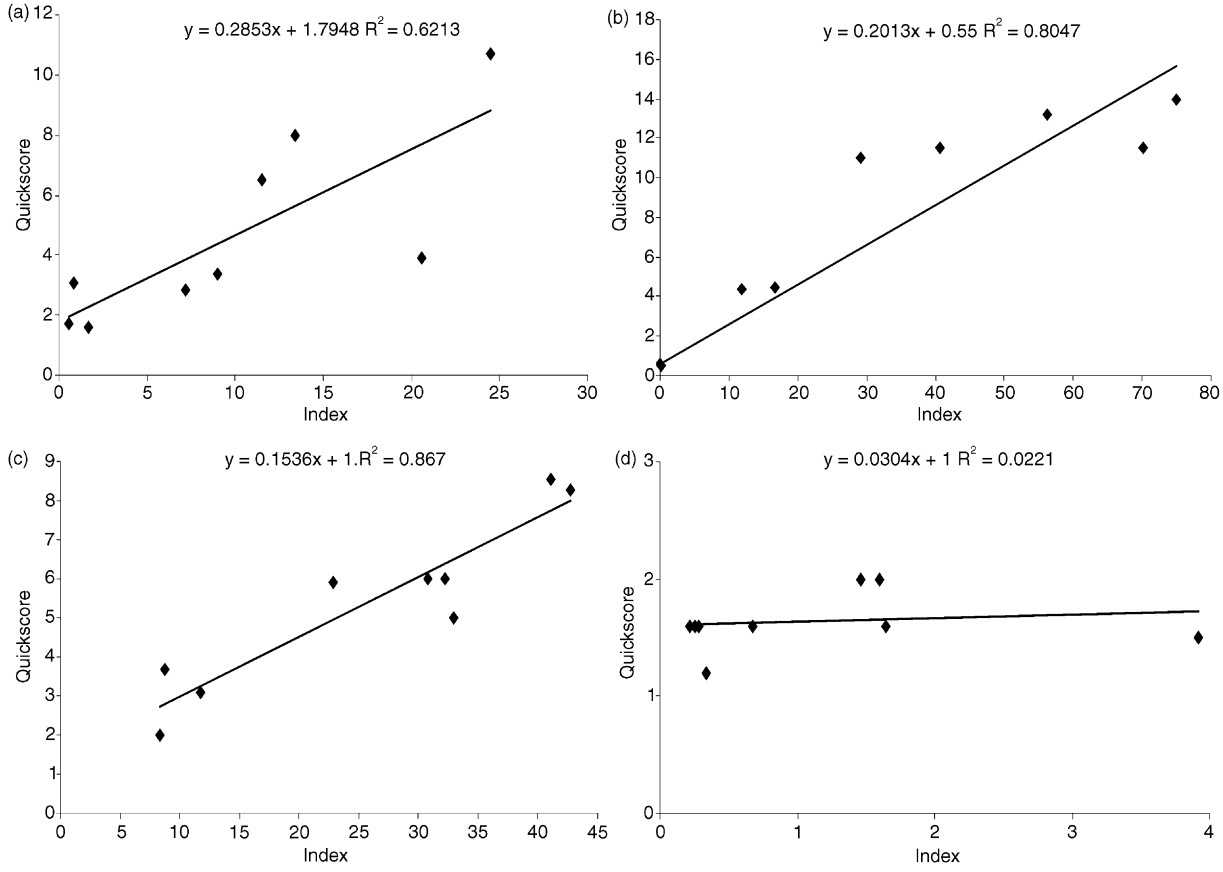


Fig. 1. Correlation between the Index and Quickcore marking methods in all tumours for (a) Ki-67, (b) p53, (c) bcl-2, (d) ApopTag.

of variation is given above each bar. The level of Ki-67 and p53 staining in individual sections of each tumour are shown in Figs. 3 and 4. Various cut-off points, ranging from 0 to 50%, are shown on the graphs derived from levels cited in a variety of immunohistochemically-based papers. This is to better illustrate the variation in staining observed between the individual tumour sections.

## 4. Discussion

The level of expression of each marker studied varies between individual sections of the tumours. The potential effect this variation would have on the interpretation of results is illustrated below.

A number of 'cut-off' points for what is to be considered positive have been suggested for Ki-67 ranging from 10% [21], to staining of any cell [22]. When applying a 10% cut-off point to the Ki-67 results in Fig. 3, five tumours would be considered positive and four negative. In four of the tumours studied, it would depend on which section was chosen as to whether it was assigned a positive or negative status. For example, evaluating section 2 with a 10% cut-off limit, three tumours would be designated positive and six negative. However, if section 4 was evaluated, five would be positive and four negative.

p53 exists in a mutant and a wild-type form [23]. The mutant form is not degraded by normal pathways and is involved in the tumorigenesis of cells. Staining indeces of 2% [22], 10% [24,25] and 50% [1] have been used as indicative of increased levels of p53 protein expression. it has even been suggested that any detectable expression of p53 is due to the mutant form [23]. If a limit of 2% is applied to the results for p53 expression (Fig. 4) then two tumours would be considered negative and seven positive. A 10% limit would designate two negative, one borderline and six positive. A 50% limit results in three overall positive and six negative.

Cut-off points of 10% [24,25] and 50% [1] have been suggested for bcl-2 expression. the tumours studied, again depending on the section assessed, would yield six positive and three borderline tumours with a 10% cut-off and no positive tumours at all with a 50% cut-off point (data not shown). Thus, the importance of choice of section as well as defining the limits for positivity can affect the interpretation of the results.

ApopTag staining also presents a similar dilemma when assigning positivity to a tumour. There is the added problem of lack of correlation between the two marking methods used. This lack of agreement is related

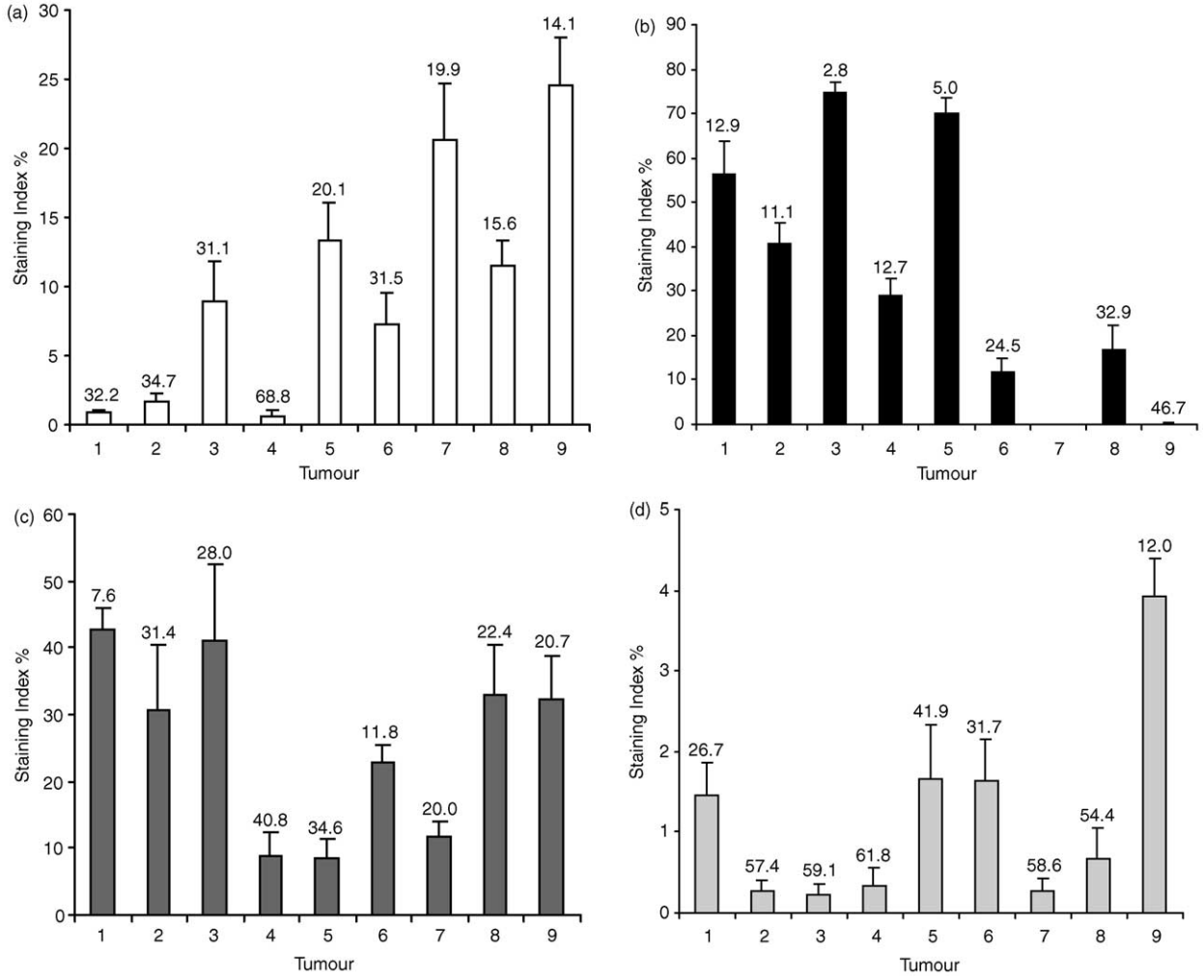


Fig. 2. Mean staining index of the various markers assayed for all nine tumours tested. Coefficient of variation (%) is displayed above each bar: (a) Ki-67, (b) p53, (c) bcl-2, (d) ApopTag.

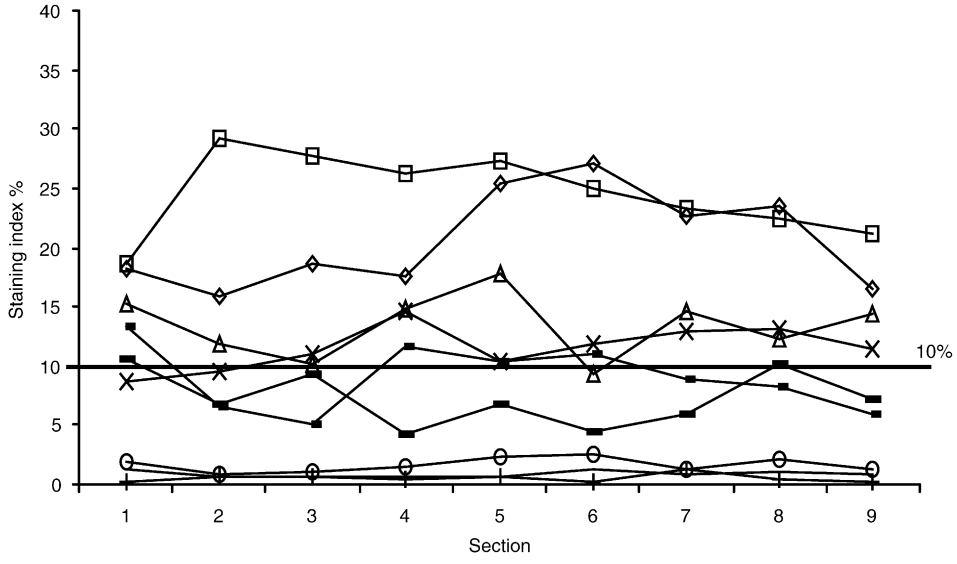


Fig. 3. Staining index of Ki-67 for all sections stained in all tumours. The line represents a 10% cut-off point for positivity, illustrating the differing results that can be obtained due to the heterogeneity of staining.

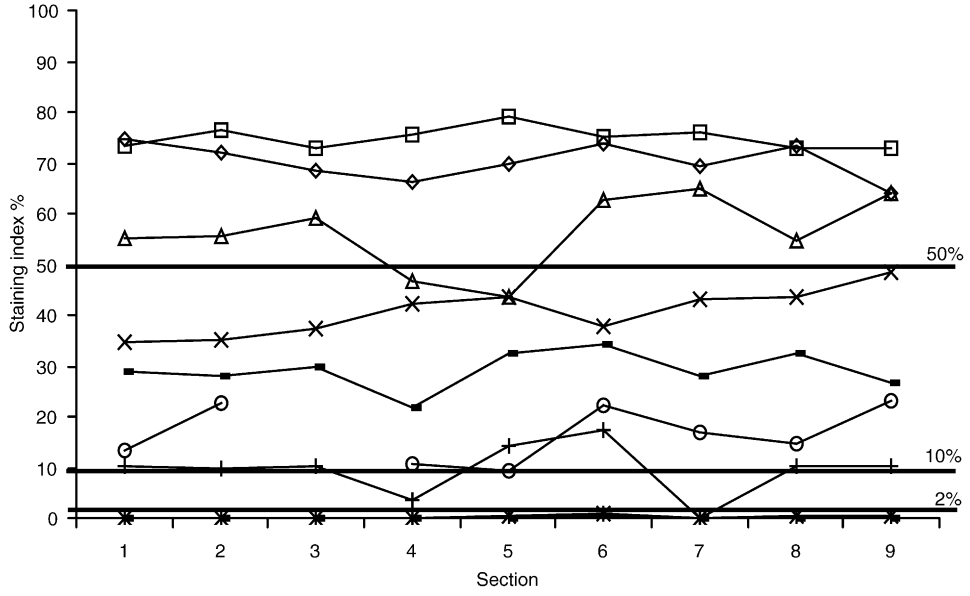


Fig. 4. Staining index of p53 for all sections stained in all tumours. The differing results that can be obtained due to the effect of both the heterogeneity in staining and the different cut-off points of 50, 10 and 2% can clearly be seen.

to the very low levels of apoptotic staining in the tumour. The Quickscore method assesses the overall staining of the section, while the staining index only assesses two random areas. Therefore, due to the low level of staining present the staining index is more likely to be negative. This results in a lower score than the Quickscore method and could account for the disparity.

In conclusion, this study shows that there is a good correlation between the staining index and Quickscore methods of assessing immunoreactivity and that tumours are heterogeneous in the expression of tumour markers.

The absence of correlation between the nine tumours and overall expression of the different markers studied is to be expected in view of the small sample size.

This study underpins the importance of standardisation of marking and staining methods and the need to stain multiple sections of tumour to obtain a more precise representation of oncogenic marker expression. To provide an even more accurate expression profile slides could also be taken from different blocks of tumour, as the observed heterogeneity is present thoughout the entire tumour not just one block. This study also provides an explanation for the limited consensus with respect to the significance and usefulness of these markers in lung cancer management.

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