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SIMPLE QUANTITATION OF IMMUNOHISTOCHEMICAL STAINING POSITIVITY IN MICROSCOPY FOR HISTOPATHOLOGY ROUTINE

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ABSTRACT

Immunohistochemical staining positivity can be quantitated in the context of conventional light microscopy. The quantitation is based on the evaluation of staining intensity of individual cells, and the fraction of positively stained cells. These variables can be combined into staining indices combining information from the fraction of stained cells and their staining intensity. The quantitated staining positivity can be successfully applied in prognostication of human cancers. The potential of these quantitation methods seems to be highest in grading of tumours of which only a small amount of tissue is available for diagnostic and prognostic purposes. This paper describes two quantitation methods and gives practical guidelines on their use in immunohistochemistry of human tumours.

Key words: immunohistochemistry, quantitation, light microscopy, MCA, CA50, bladder cancer, breast cancer, prognosis

INTRODUCTION

Quantitation of immunohistochemical staining is valuable, especially in the light of evidence relating the behaviour of neoplasm with the intensity of staining of certain cancer markers (Baak et al. 1991, Malmström et al.1988, Eskelinen et al. 1990b, Eskelinen et al. 1992, Lipponen et al. 1990a,b, 1991a,1992, Tervahauta et al. 1991). Such an association has recently been shown i.e. by Baak et al. in their study on the staining of the HER-2/neu oncogene product in breast cancer (Baak et al. 1991). DNA cytometry is an example of quantitative histochemistry in which the intensity of the Feulgen stain is related to the amount of DNA in the nuclei, and to the ploidy of the cell (Fossâ et al. 1977). We know that DNA ploidy is a predictor in many types of neoplasms (Blomjous et al. 1989, Eskelinen et al. 1990b).Traditionally the intensity of immunohistochemical staining has been graded as positive or negative,but our group has used another

approach and tested more detailed quantitation (Aalto et al. 1982, Eskelinen et al. 1990b,c,1992, Lipponen et al. 1990a,b 1991a, 1992). Such an approach is related to the analysis of the results of blood tests, which may show variation in efficiency dependent on the way the results are interpreted, or combined (Eskelinen et al. 1988). We started to test the quantitative evaluation of CEA immunohistochemistry in 1982 and found that such approach is more reproducible than subjective evaluation (Aalto et al. 1982). Our approach was based on point counting principles and took often quite a lot of time. We also included the subjectively evaluated staining intensity. However, it was noticed that subjective evaluation could be made better by considering different aspects of staining intensity in the microscopic image, and each aspect in turn. Recently this has led to a number of studies which clearly demonstrated the value of detailed quantitation in prognosis (Eskelinen et al. 1990b,c, 1992, Lipponen et al. 1990a,b, 1991a). It was also shown that the value of staining in prognostication may be dependent on the way the results are interpreted. This paper describes two simple quantitation methods of immunohistochemical staining and reviews some recent results with these methods in prognostication of cancers.

QUANTITATION OF IMMUNOHISTOCHEMICAL STAINING POSITIVITY

The immunohistochemical staining may vary considerably within individual samples. Accordingly it is necessary to get an overall impression of the staining result before a more detailed analysis. The analysis can be done in several ways. In this paper two methods are described. In the first method (Method I) four variables are evaluated, and in the second (Method II) two variables are recorded. The basic elements of both of these methods are the same - the evaluation of the staining intensity of an individual cell, and the evaluation of the fraction of positively stained cancer cells in the sample. The staining intensity can be graded into four grades using arbitrary units 0, 1, 2, 3 in the order of increasing staining intensity. In the evaluation, 0 corresponds to no positive (i.e. negative) staining, 1 to slight or weak staining, 3 to heavy or marked staining and 2 to intermediate staining between 1 and 3. The fraction of positively stained cells is expressed as the fraction of positive cells, i.e. the number of positively stained cells divided by the total number of cells. The evaluation can be done in the field of an eyepiece graticule or in the entire microscope field. When the fraction of positively stained cell in the entire section is evaluated, several fields are studied, and the average fraction of positive cells is recorded for further analyses. Estimation of the fraction at an accuracy of 5 % seems to be sufficient for prognostic purposes.

Method I

In this method four variables describing the immunoreactivity are recorded and they are:
1. The fraction of positively stained cancer cells in the entire section (FPtot), in percent.
2. The average staining intensity of cancer cells in the entire section (ASItot).
3. The fraction of positively stained cancer cells in the maximally stained area (1-2 microscopic fields, objective magnification 40x) of the sample (FPmax), in percent.
4. The average staining intensity of cancer cells in the area (1 microscopic fields, objective magnification 40x) of maximum staining intensity (ASImax).

Method II

In this method only two varibles are recorded. The staining intensity (i) of cells in the sections is scored using arbitrary units 0, 1, 2 and 3 in the order of increasing staining intensity as described above. The fraction (f) of cells of each staining intensity in the entire section is also evaluated as detailed earlier. Thereafter a staining index (I) is calculated using the formula:

$$I = 0 * f1 + 1 * f2 + 2 * f3 + 3 * f4 \quad (1)$$

where f1-f4 correspond to the fraction of cells of each staining intensity. The staining index describes the staining in the entire section and can be used in further analyses.

A PRACTICAL QUIDE FOR THE QUANTITATION OF IMMUNOHISTOCHEMICAL STAINING POSITIVITY

The entire section is first screened using an objective magnification of 4x or 10x to get an overall impression of staining. While screening the sample one should identify possible necrotic areas in a section since these areas should not be included in the evaluation due to the risk of artefactual staining. Thereafter the fraction (%) of positively stained cancer cells (FPtot) is estimated using objective magnifications of 10x, 25x and 40x and the result is recorded. While the fraction of positively stained cancer cells is recorded one also estimates the average staining intensity of positively stained cancer cells (ASItot). Usually the staining varies in different parts of the sample. However, a subjective estimate of the average staining intensity of cancer cells can be given expediently. Thereafter one looks for maximally stained region in the sample. The maximally stained area should cover at least 1 microscope field (diameter 450 µm; objective magnification 40x). In this maximally stained area the fraction of positively stained cancer cells (FPmax), and the staining intensity of

positively stained cancer cells (ASImax) are estimated separately. The results can be most conveniently recorded on a data collection sheet. If one prefers to use the method II the fraction of cells in each staining positivity category in the sample is recorded. This can be done over the whole section, or separately over the most intensively staining area.

DISCUSSION

The potential of immunohistochemistry in identifying specific cell types has been extensively studied in histopathology. Grading of malignant lesions based on expression of certain tumour markers has been particularly helpful in haematology (Heckner 1981). Recent results clearly indicate that the expression of tumour markers has predictive value also in other human tumours (Eskelinen et al. 1990b,1992, Lipponen et al. 1990a,b,1992). The demonstration of prognostic value for tumour markers is, however, difficult due to several sources of variation. Variation sources include factors related to processing of samples (Miettinen 1989, Pettigrew 1989, Wold et al. 1989), intratumoural heterogeneity (Lipponen et al. 1991a) and observer variations (Aalto et al. 1982, Kosma et al. 1985). Factors related to processing of samples have been recently reviewed (Miettinen 1989) and this paper gives practical advice in the simple quantitation of immunohistochemical staining positivity for histopathology routine.

Usually the results of immunohistochemical staining are reported as positive or negative. The method, however, is crude and a substantial amount of prognostic information may be omitted. In the analysis of c-erbB-2 oncoprotein expression only heavy or marked expression has predictive value whereas tumours with a low expression can not be distinguished from tumours totally negative for c-erbB-2 oncoprotein (Baak et al. 1991, Tervahauta et al. 1991). Neither can bladder tumour cells with weak positivity for C-50 antigen be distinguished from cells totally negative for C-50 antigen (Lipponen et al. 1992). In such situations the estimation of the fraction of positively stained cells will allow grading of samples with varying numbers of detectably positive cells.

The quantitation method I includes both continuous (FPtot, FPmax) and graded variables (ASItot, ASImax). In association with bladder tumours both of the assessments give prognostic information in terms of progression and survival (Lipponen et al. 1990a, 1991a). Also the predictive value of PCNA/cyclin immunolabeling over the entire section in bladder tumours corresponds with the predictive value based on the estimate at the region of maximum staining (Lipponen et al. 1992). However, the simultaneous assessment of FPtot and

FPmax may help in avoiding the influence of staining artefacts. Method II allows the use of statistical tests suitable for analysis of continuous variables and several group limits can be tested in a continuous scale. There is still much work to be done for finding the optimal cutpoints for prognostic purposes. Another advantage of the method II is the simplicity of the assessment.

Today malignant tumours are diagnosed at an earlier phase than previously, and thus tumours are smaller and the amount of cancer tissue available for diagnostic and prognostic purposes may be limited. The counting of mitotic figures (Haapasalo et al. 1989) in a small biopsy specimen need not be representative for the whole neoplasm. The estimation of DNA ploidy or the S phase fraction (SPF) by flow cytometry in such samples may be problematic due to a small number of neoplastic cells. Additional information gained through quantitation of immunohistochemical staining positivity may be helpful in prognostication under such conditions (Lipponen and Eskelinen 1992).

The quantitation methods of immunohistochemical staining introduced in this report have led to encouraging prognostic results in retrospective cohorts of patients with neoplastic diseases. The quantitative assessment of mucinous carcinoma antigen (MCA) in breast cancer predicts recurrence free survival efficiently, particularly in premenopausal women (Eskelinen et al. 1990b). In accordance with the above, the quantitative assessment of MCA in transitional cell bladder tumours is able to identify those patients with an increased risk of pelvic lymph node involvement and distant metastasis (Lipponen et al. 1990a). A similar favorable relationship was found in a quantitative assessment of the expression of tissue polypeptide antigen (TPA) and recurrence free survival in a prospectively followed up cohort of breast cancer patients (Eskelinen et al. 1992). However, in pancreatic adenocarcinoma quantitation of MCA could not predict survival (Eskelinen et al. 1990c). This result is probably associated with the extremely aggressive nature of pancreatic adenocarcinoma.

Blood group antigens alone or in combination with other quantitative variables carry significant prognostic information in transitional cell bladder tumours (Malmström et al. 1988). Quantitative assessment of a blood group antigen related Lewis antigen (Lipponen et al. 1990a, 1991a, 1992) gives significant prognostic information in transitional cell bladder tumours. The prognostic value of C-50 antigen expression in superficial tumors is particularly significant. In a cohort of 130 Ta-T1 transitional cell bladder tumours the quantitative assessment of C-50 antigen expression was the most important independent predictor in a multivariate analysis (Lipponen et al. 1992). The analysis in-

cluded clinical stage, mitotic indices, morphometric variables and flow cytometric data (DNA ploidy, SPF).

The assessment of breast cancer nuclei positive for estrogen receptors (Helin et al. 1989) can be based on the principles depicted in this report and the results from subjective measurements are in accordance with those done by image analysis. Recent analyses of growth fraction in epithelial neoplasms have applied the same quantitation principles (Bush et al. 1991). The assessment of Ki-67 immunolabeling in bladder cancer is highly reproducible and relevant, and parallels the findings of PCNA/cyclin immunostaining in TCC (Lipponen et al. 1992). In the latter analysis the fraction of proliferating cells well correlated with the established proliferation indices.

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