Virchows Arch (2002) 440:325–329
DOI 10.1007/s004280100539

SHORT COMMUNICATION

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Fast and sensitive immunodetection of carcinoma cells in sentinel nodes

Received: 3 August 2001 / Accepted: 9 August 2001 / Published online: 28 September 2001

Abstract In a number of clinical situations, especially in the context of the recent sentinel node concept, lymph-node involvement has to be determined intraoperatively. Since serious and dependable decisions are to be made according to the result of this examination, the most reliable method for the detection of tumour cells should be applied. We and others have shown previously that routine histological examination underestimates lymph-node metastases, and that immunohistochemistry (IHC) significantly improves the accuracy of staging. However, IHC has so far been difficult to apply to the intraoperative examination of cryosections since it has required too much time. We have developed a novel modification of IHC for the rapid detection of metastases of carcinomas in cryosections from lymph nodes. It is based on a unique directly labelled cytokeratin antibody, immunofluorescence, and a specially devised staining solution. This one-step staining procedure can be performed within 10 min. At the same time, its sensitivity is very high. Single tumour cells can easily be detected, and background staining is very low. The high sensitivity could result in a markedly improved reliability of sentinel node-based decisions.

Keywords Lymph node staging · Quick section · Immunohistochemistry · Cytokeratin · Intraoperative diagnosis · One-step staining

Introduction

The histological examination of lymph nodes is an essential part of the TNM classification of carcinomas. We demonstrated more than 10 years ago that the application of immunohistochemistry (IHC) leads to the detection of about 10% additional cases with lymph-node involvement relative to conventional staining [25, 26]. This was confirmed in numerous studies of different types of cancer. Most of the additionally detected metastases consist of small groups of tumour cells. Data available so far are consistent with the assumption that they are of clinical relevance [5, 17].

In a number of clinical cases, e.g. in testicular cancer, lymph nodes have to be examined during surgery, and the result determines how to proceed with the operation [27]. A dramatic increase in intraoperative node examinations can be expected with the emerging sentinel node concept in breast and other cancers [1, 2, 3, 5, 8, 9, 15, 16, 17, 18, 27, 28]. In these cases, in which the result is crucial for the decision making of the surgeon, both the experience of the pathologist and the most reliable detection technique are of utmost importance. So far, however, IHC was not readily applicable to the intraoperative examination of cryosections because it required too much time. A first attempt to apply IHC to quick section diagnosis was undertaken by us in 1992 [24, 27]. Recently, several procedures were published with the same aim [10, 20, 22]. We now propose a novel immunostaining protocol specially devised for this purpose which is highly reliable and can be performed in about 10 min. Its main features are the application of direct labelling, the employment of the immunofluorescence technique, an extremely well-suited monoclonal anti-cytokeratin (CK) antibody, and a specially devised staining solution. This protocol is given in Table 1. In this communication, we outline the rationale of choices made and the results of experiments aimed at optimising the procedure. Finally, we briefly discuss the advantages, limitations and possible fields of application of the method. We have not dealt with topics such as the number and position of sections to be selected or the minimal size of tumour cell clusters considered clinically relevant.

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Table 1 Staining protocol. *PBS* phosphate-buffered saline, *TRITC* tetramethylrhodamine B isothiocyanate, *UV* ultraviolet

Working steps

1 Prepare cryostat sections (4-µm thick) on microscope slides
2 Add staining solution to the dry, unfixed sections for 7 min at room temperature
3 Wash three times with PBS
4 Mount with PBS or another suited mounting medium
5 Examine under an epifluorescence microscope at an excitation wavelength of around 550 nm (TRITC filter set). For visualising the counterstained nuclei, switch to UV excitation (around 350 nm)

Staining solution

Monoclonal antibody A45-B/B3-Cy3, 20 µg/ml in PBS, with 0.2% Triton X-100 and 0.5 µg/ml DAPI

Notes

The staining solution should be freshly prepared on the day of use from the following stock solutions. A45-B/B3-Cy3 (50×): 1 mg/ml A45-B/B3-Cy3 in water, with 0.1% Na-azide; stored at +4°C in the dark, stable for at least 1 year. DAPI (1000×): 500 µg/ml 4′,6-diamidino-2-phenylindole in water; can be kept at +4°C in the dark. The addition of DAPI, which selectively stains nuclei, is optional but highly recommended for a better recognition of the nodal tissue structure. Fixation of the cryosections with acetone (10 min at room temperature) is possible but not necessary. PBS as mounting medium is only intended for immediate examination; otherwise, mounting media with 50% glycerol are recommended. Sustainable mounting is possible with Mowiol [21]

Choice of antigen

CKs are still the most reliable marker for the detection of metastases of carcinomas in non-epithelial tissues such as lymph nodes or bone marrow, despite the presence of very small amounts of CK 8 and CK 18 in reticular cells [7]. It is, however, essential not to rely on an antibody specific for a particular CK, since the expression of a single member of the CK family may be downregulated in certain tumours.

Choice of antibody

The monoclonal antibody (mAb) A45-B/B3 (mouse IgG1,κ) is a unique pan-epithelial CK antibody [12, 19, 23]. Although it was classified as a CK 18 antibody during the TD5 Workshop [23], own data show that it does also bind to other CKs. This mAb has been employed in extensive immunohistological studies, in which it was found to react with all CK-containing cells (independently of the presence of CK 18) and was instrumental in the detection of CK in unusual locations [13, 14]. It was found to bind faster than three other commercial pan-CK antibodies to sections ([24] and unpublished results) and proved very powerful in the detection of micrometastases of carcinomas in lymph nodes [25] and in bone marrow [4]. This antibody was provided as a Cy3-labelled conjugate (lot PH1750; 1.16 moles dye per mole antibody) by Micromet (Martinsried/Munich, Germany).

Choice of dye

Epifluorescence is highly sensitive and well suited for the detection of rare positive sites in histological sections provided that background fluorescence is low. From the increasing number of available fluorescent dyes, Cy3 (indocarbocyanine 3.18, M_r 949, excitation maximum 553 nm; emission maximum 575 nm) [6] was chosen for a number of reasons. First, its fluorescence intensity is very high [about 100 times that of fluorescein isothiocyanate (FITC)]. Second, fading is minimal. Third, it is a hydrophilic dye which shows almost no unspecific tissue binding. Finally, at its excitation wavelength (around 550 nm), autofluorescence of lymph-node tissue is negligible in contrast to the situation in the FITC excitation range (around 490 nm). Filter sets devised for TRITC (tetramethylrhodamine B isothiocyanate) are well suited. Texas-red filter sets are not recommended. We employ an Axiophot fluorescence microscope (Zeiss, Jena, Germany) equipped with a digital camera (CF20DXC, Kappa Meßtechnik, Gleichen, Germany), but any other fluorescence microscope with a TRITC filter set would be suited. Observations were made with a 40× lens without oil immersion. The following parameters were varied in order to optimise the staining protocol.

Fixation

Unfixed and acetone-fixed lymph-node sections (10 min at room temperature) were treated with staining solutions containing or lacking Triton X-100. In the presence of 0.2% Triton (Sigma, Deisenhofen, Germany), staining was only marginally different (slightly more intensive after fixation). Without Triton, unfixed sections showed intolerable unspecific binding. We concluded that in the presence of Triton fixation can be omitted.

Dye concentration and time of staining

It is obvious that in short-term incubations both parameters are interdependent. This was extensively examined with unfixed lymph-node sections. The following data

were obtained. First, a minimal penetration time of 5 min is required for 4-µm sections. Second, for 10 min staining, a concentration of 5 µg/ml antibody A45-B/B3 is sufficient. Third, by enhancing the antibody concentration to 10 µg/ml, incubation time can be shortened to 7 min. Fourth, the staining intensity can be further enhanced without unspecific binding by increasing the antibody concentration to 20 µg/ml. For the sake of maximum sensitivity, we recommend this antibody concentration for the standard protocol, although 10 µg/ml may suffice.

Counterstaining

Due to the low background staining of A45-B/B3-Cy3 and the lack of autofluorescence at the excitation wavelength of 550 nm, the structure of the uninvolved lymph-node tissue is barely visible except for a faint CK staining of reticular cells (Fig. 1c). Therefore, it is desirable to counterstain the nodal tissue. This is done by staining the nuclei with 4′-6-diamidino-2-phenylindole (DAPI; Sigma; 0.5 µg/ml added to the staining solution; Fig. 1b). We consider it of advantage that the fluorescence of the nuclei can be switched on and off by changing the filter blocks between TRITC and ultraviolet. For a demonstration of the counterstaining with DAPI, Fig. 1b was produced with a special triple filter (AF Analysentechnik, Tübingen, Germany), which allows the concomitant visualisation of DAPI, FITC, and Texas-red staining. Alternatively, due to the fact that there is always some autofluorescence at FITC excitation, the tissue structure can be made visible without counterstaining by switching to FITC excitation.

As an example, lymph-node cryosections from breast cancer patients stained according to the staining protocol as outlined in Table 1 are shown in Fig. 1. CK-positive carcinoma cells (Fig. 1a) are clearly recognisable against an almost zero background fluorescence of mesenchymal lymph node cells, which are either CK negative or (in case of reticular cells) weakly positive (Fig. 1c). Moreover, the morphological appearance of reticular cells and their CK filaments is strikingly different from that of carcinoma cells, as is clearly visualised in immunofluorescence. The use of fluorescence microscopy may appear unusual for routine histology, but its advantage in detecting small objects is so evident for the intended purpose that it outweighs by far any inconvenience connected with the change of the technique. Compared with indirect IHC, it is much easier to perform and less costly with respect to the staining reagents. Direct immunofluorescence with an antibody to glial fibrillary acidic protein (GFAP) has been successfully employed for the intraoperative diagnosis of neurosurgical tumours [10]. Apart from the diagnostic goal and the use of target antigen, our staining protocol differs from that of these authors by omission of fixation and blocking steps, and addition of Triton X-100 to the staining solution.

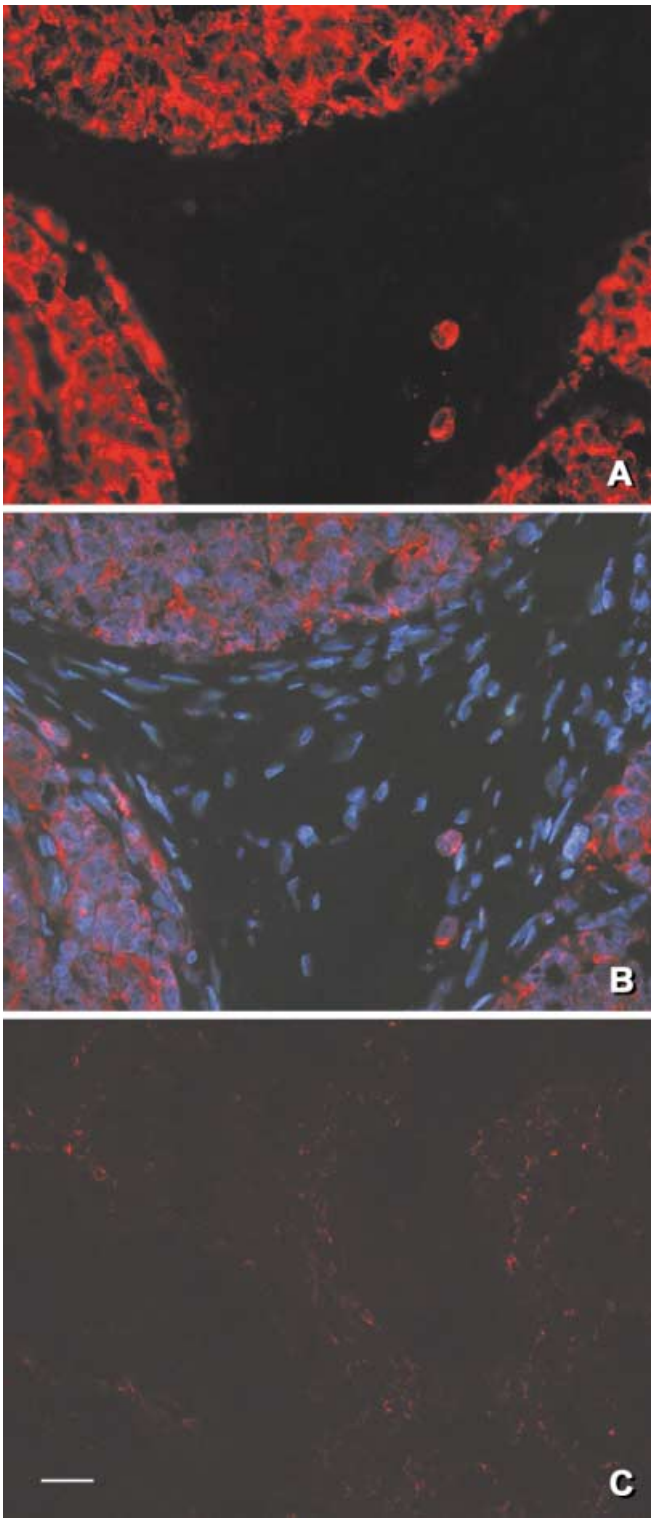

Fig. 1 Cryosections of lymph nodes from breast cancer patients stained according to the staining protocol outlined in Table 1. **a**, **b** Heavily involved node with cytokeratin-positive metastases and two single tumour cells (*red*); **b** counterstaining of the nuclei with 4′-6-diamidino-2-phenylindole (DAPI) is shown (in this case a special triple-filter set DAPI/fluorescein isothiocyanate/Texas red was used, which is not optimal for Cy3). **c** Metastasis-free node showing weak cytokeratin staining of reticular cells. Magnification ×375 (bar 20 µm)

Staining protocols for intraoperative IHC that are not based on immunofluorescence [20, 22, 27] require more time in any case, even with the elegant EPOS (enhanced polymer one-step staining system) procedure, which, in fact, is a two-step procedure since it requires the addition of the substrate. We also consider the fact that the Cy3 label is much smaller than peroxidase an advantage of our procedure.

The sentinel node concept relies on fast and dependable examinations of the primary draining lymph node of a given tumour during surgery. With the described method, the highest possible sensitivity (one tumour cell per section) is easily achieved. As a consequence, depending on the number of sections per node examined, the diagnosis can be given with unsurpassed reliability. Since there is at present some controversy about sentinel node-based decisions in surgery [11, 16], we would argue that a better methodology of nodal examinations, and especially more dependable NO diagnoses could lead to better results with the application of this surgical concept. From the technical point of view, the proposed procedure proved to be simple, fast, sensitive and very satisfying. It is presently being evaluated in three laboratories.

The antibody employed in the present modification is already in use in the immunohistochemical detection of tumour cells in bone marrow [4]. There is every reason to assume that the Cy3-labelled antibody in combination with fluorescence detection will also be applicable to advantage for this purpose. Since non-epithelial tumour cells do not contain CK, the present procedure is only applicable to carcinomas. In case of non-epithelial tumours [3], an analogous procedure with a directly labelled antibody to another relevant tumour marker could be tried.

Acknowledgements The technical assistance of Ms. A. Koberling is gratefully acknowledged. Cy3-labelled antibody A45-B/B3 was kindly provided by Micromet (Martinsried/Munich, Germany). We are also thankful to Prof. P.M. Schlag (Robert Rössle Clinic, Charité, Campus Buch, Humboldt University, Berlin), for his continuing interest and for a series of cryosections of lymph nodes from breast cancer patients.

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