# Rapid immunohistochemical detection of tumor cells in gastric carcinoma

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**Abstract.** The detection of single tumor cells or tumor cell clusters represents an important issue in intraoperative frozen section analysis. For example, surgical margins may be evaluated in order to minimize the number of additional operations. Furthermore, intraoperative diagnosis of lymph node micrometastasis (LNM) may help to define the area of appropriate lymph node dissection. In addition to haematoxylin and eosin (H&E)-stained sections, immunohistochemical detection of single tumor cells or cell clusters may be helpful in this context. The aim of this study was to evaluate the clinical significance, reliability and sensitivity of intraoperative rapid immunostaining of frozen sections. Therefore, we compared the results of rapid immunohistochemical staining of frozen sections and paraffin sections applying the EnVision™ and Histofine® detection systems. In a prospective immunohistochemical study, paraffin and frozen sections of 20 gastric cancer specimens were analyzed. Paraffin as well as frozen sections were stained immunohistochemically applying the EnVision and Histofine detection systems. As primary antibodies, AE1/AE3 (anti-cytokeratin), EMA (anti-MUC1) and B lymphocyte marker anti-CD20 were applied. The rapid immunostaining procedure was able to be completed within 10-13 min. Rapid immunohistochemical staining of frozen and paraffin sections of the same tumors resulted in comparable immunoreactivity. The rapid EnVision and Histofine procedures allowed immunostaining of frozen sections in less than 13 min. These methods can represent useful additional tools in routine surgical pathology and research, enabling a more accurate frozen section diagnosis compared to staining with H&E alone. Intraoperative rapid immunostaining can be a simple and useful technique to detect LNM.

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## Introduction

The detection of single tumor cells or small tumor cell clusters is of utmost importance in intraoperative frozen section analysis. For example, surgical margins of gastrectomy specimens may be investigated in order to exclude tumor infiltration. For undifferentiated tumors and very small lesions, e.g. micrometastases, a diagnosis cannot be made on the basis of H&E staining alone, but only after an additional, time-consuming, immunohistochemical investigation of paraffin-embedded tissue. Recently, the sentinel node concept has been introduced into the surgical procedure for gastric cancer (1-3). The rapid, reliable and sensitive intraoperative histopathological evaluation of distant sentinel lymph nodes represents an important issue for the successful application of the sentinel node concept (1). Therefore, a dramatic increase in intraoperative node examinations can be expected as a result of the emerging sentinel node concept (4-13). Since an intraoperative histopathological diagnosis is crucial for the selection of the appropriate area of lymph node dissection, both the experience of the pathologist and the most reliable detection techniques are very important.

Reported methods facilitating the detection of nodal micrometastases include immunostaining (IHC) (14-18). Turner *et al* (19) observed that 10 (14.3%) of 70 patients who were assessed as tumor-free by haematoxylin and eosin (H&E) staining, were found to be sentinel node-positive by immunohistochemical cytokeratin (CK) analysis. Natsugoe *et al* (20) reported that 14 (52%) of 27 patients who underwent D2-lymphadenectomy for gastric cancer were found to be lymph node-positive by CK immunohistochemistry. The prognosis for node-positive patients was worse compared to lymph node-negative patients. So far, however, standard IHC was not readily applicable to the intraoperative examination because it required too much time (4-6 h). However, according to techniques developed during the last few years, the intraoperative immunohistochemical examination of frozen sections has become possible allowing for a histopathological diagnosis to be conducted within 10-15 min (21-30). However, most of the methods described exhibited certain disadvantages, such as a time-consuming procedure (≥20 min) or the requirement of directly labelled primary antibodies. However, by applying microwave treatment and an HRP procedure, Ichihara *et al* (24) were able to immunostain

frozen sections intraoperatively within 13 min. Recently, the immunohistochemical detection systems EnVision™ and Histofine® have become commercially available, and the EnVision system has been described as a very sensitive and rapid detection system in IHC (31,32). The aim of the present study was to evaluate the clinical significance, reliability and sensitivity of these methods. Therefore, we compared the staining of frozen as well as paraffin sections applying the EnVision and Histofine detection systems.

## Materials and methods

In the immunohistochemical study, paraffin and frozen sections of 20 gastric adenocarcinoma specimens were analyzed applying the EnVision and Histofine detection systems. In frozen sections, rapid immunohistochemical staining was also performed by using the EnVision and Histofine detection systems.

*Tissue specimens and preparation*. Parts of the tumor specimens were fixed in 10% formaldehyde and embedded in paraffin. Sections, 4-$\mu$m thick, were cut from the paraffin blocks containing representative histological features from the periphery, the center, and the invasion front of the tumor (three slides per patient). The tissue sections were deparaffinized and dehydrated, followed by microwave treatment for 10 min at 750 W for antigen retrieval.

The other parts of the tissue samples from the surgical specimens were freshly frozen in liquid nitrogen for 30 sec immediately after removal and then transferred to a cryostat (Leica, Bensheim, Germany). Serial frozen sections (4 $\mu$m) were cut, air-dried for 30 sec, fixed in acetone at room temperature (22˚C, RT) for 1 min and air-dried for 15 sec at RT, before being subjected to the staining procedure.

*Choice of antibodies*. The specimens were incubated with a mixed solution of monoclonal mouse anti-human cytokeratin antibodies (AE1/AE3) (DakoCytomation, Hamburg, Germany) at a dilution of 1:50. This antibody cocktail (AE1/3) is known to detect a broad spectrum of cytokeratins. Furthermore, MUC1, a transmembrane protein characterized by a large extracellular tandem repeat domain and expressed on the apical surface of most glandular and ductal epithelial cells (33) was analyzed by mab EMA (Klon E 29, Dako Cytomation). As an additional positive control, mab CD20 (Klon 26, DakoCytomation), a B lymphocyte marker, was applied.

*EnVision and Histofine IHC procedures*. The EnVision-HRP system (DakoCytomation, no. K4000) applied in this study represents a two-step method. The application of the primary antibody is followed by a polymeric conjugate consisting of a large number of secondary antibodies (goat anti-mouse or goat anti-rabbit) bound directly to a dextran backbone containing HRP. One such conjugate contains up to 100 HRP molecules and up to 15 antibodies. Histofine Simple Stain MAX PO (M) (universal immunoperoxidase polymer, anti-mouse) received from Medac, Hamburg, Germany, is a labelled polymer prepared by combining amino acid polymers with peroxidase and goat anti-mouse Ig which are reduced to Fab' fragments.

The sections were incubated with the primary antibody in 'antibody diluent' (DakoCytomation) for 3 min. Subsequently, goat-anti-mouse or goat anti-rabbit EnVision-HRP-enzyme conjugate were also incubated for 3 min, respectively. The 'highly sensitive diaminobenzidine plus' (DAB+) and the 3-amino-9-ethylcarbazol plus (AEC+) chromogens (both from DakoCytomation) were used as substrates for the EnVision-HRP-enzymes. Staining intensity was further enhanced (modifying the manufacturer's protocol) by performing all incubation steps (primary antibodies, EnVision, and substrate reactions) on slides placed horizontally on a thermal plate at

Table I. Clinical and pathological data.

| Factor | Category | Cases (n=20) |
|---|---|---|
| Sex | Men | 12 |
| | Women | 8 |
| Age (mean ± SD) | | 68.1 |
| Lauren type | Intestinal | 5 |
| | Diffuse | 9 |
| | Mixed | 6 |
| Grading | 1 | 0 |
| | 2 | 6 |
| | 3 | 12 |
| | 4 | 2 |
| Depth of tumor invasion | T1 | 0 |
| | T2 | 6 |
| | T3 | 13 |
| | T4 | 1 |
| N-status | N0 | 7 |
| | N1 | 4 |
| | N2 | 5 |
| | N3 | 4 |
| M-status | M0 | 17 |
| | M1 | 3 |

Table II. The time required to perform H&E and immunohistochemical examinations.

| | A1/A3 / mab EMA / mab CD20 examination | | |
|---|---|---|---|
| | Routine IHC | EnVision™ | Histofine® |
| Approximate time required | 4-6 h | 10-13 min | 10-13 min |

IHC, immunohistochemistry; H&E, hematoxylin and eosin.

Table III. Semiquantitative score (0-2) of immunohistochemical examination.

| | Paraffin sections | | | | | | Frozen sections | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | EnVision™ | | | Histofine® | | | EnVision™ | | | Histofine® | | |
| | EMA | AE1/AE3 | CD20 | EMA | AE1/AE3 | CD20 | EMA | AE1/AE3 | CD20 | EMA | AE1/AE3 | CD20 |
| Score 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Score 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 5 | 0 | 4 | 5 | 0 |
| Score 2 | 20 | 20 | 20 | 20 | 20 | 20 | 19 | 15 | 20 | 16 | 15 | 20 |

37˚C. After each incubation, the slides were dipped in TBS or, after the substrate reaction, in tapwater at RT and waved at maximum speed for 10 sec. Excess liquid (buffer/water) was soaked up by a paper towel. After the last washing in tapwater, the slides were dipped quickly in distilled water before counterstaining with Meyer's haematoxylin (Sigma, 15 sec), followed by 30 sec in 42˚C tapwater. The sections were mounted in Aqua Tex (Merck, Darmstadt, Germany) and examined microscopically. The same procedure was performed using the Histofine detection system instead of the EnVision system. Staining was evaluated by two independent observers taking into account the results obtained with the standard IHC procedures (HRP, LSAB). The evaluation was performed semiquantitatively (score 0-2; 0=no activity, 1=good immunoreactivity, 2=strong immunoreactivity).

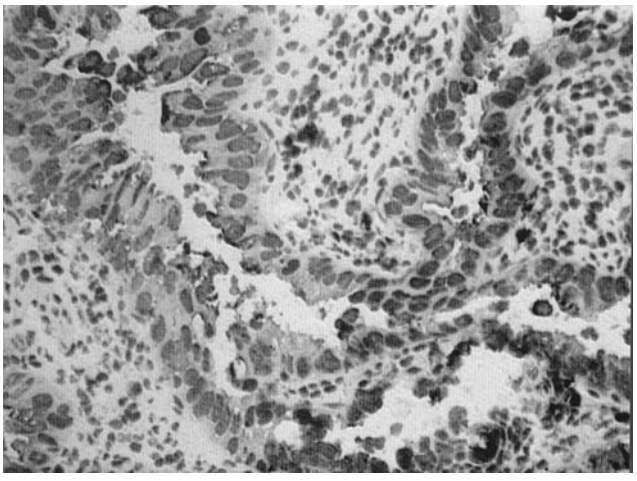

Figure 1. Positive immunoreactivity for MUC1-EMA (tubular gastric adenocarcinoma).

## Results

The clinicopathologic features of the 20 patients are summarized in Table I. The time required for rapid immunostaining of frozen sections was approximately 10-13 min, each for the EnVision and the Histofine detection systems (Table II). The rapid EnVision and Histofine procedures resulted in strong and specific staining applying primary antibodies directed against cytokeratin, MUC1 and CD20 antigen (Fig. 1). The immunoreactivity was evaluated semiquantitatively (graded from 0-2). As shown in Table III, the results of the rapid immunohistochemical staining of the frozen sections were similar to those achieved by conventional immunostaining in paraffin sections. Non-specific (background) staining was not observed or only very faintly.

## Discussion

Intraoperative frozen section analysis must be accurate and rapid. An additional rapid immunohistochemical procedure may help in the histogenetic classification of tumors as well as the evaluation of surgical margins. In addition, it may be helpful for the diagnosis of lymph node metastasis. Recently, the clinical significance of micrometastasis in lymph nodes, blood, bone marrow, ascites and pleural effusion has been reported (3,17,34-39). In particular, the presence or absence of lymph node metastasis is helpful in determining the appropriate area for lymphadenectomy. It is unknown whether such cancer cells are viable in lymph nodes, although a study by Scheunemann *et al* (40) suggested that cancer cells detected by immunohistochemistry were viable and had metastatic potential. Sentinel node navigation surgery could be employed to ascertain the presence or absence of LNM during operation. If LNM is found by rapid immunostaining, it would be necessary to extend the area of lymphadenectomy. Similarly, in patients with advanced cancer, distant lymph nodes should be removed if LNM is found in regional lymph nodes. In patients with carcinoma of the oesophagus and stomach, the reported prognosis was worse in those with LNM (35,41-44).

The time required for routine immunohistochemistry is approximately 2-4 h, whereas the time required for rapid immunohistochemistry applying the reported methods using the EnVision kit/HRP or the Histofine kit is approximately 10-15 min, a time considered appropriate for intraoperative pathological assessment. Several studies have tried to reduce the time for immunostaining using various different techniques (21,24,29). However, some of the methods described still require 20 min or more to perform (45,46). In a study by Matsumoto *et al* (44) LNM could be diagnosed in approximately 30 min by rapid immunohistochemistry. Dabbs *et al* (22) reported a rapid ABC method that took 20 min or less, but only cytological specimens were investigated. Many studies have described the use of directly labelled primary antibodies to reduce the time for immunostaining of frozen sections to 7-15 min (21,27,28,30). However, directly labelled primary antibodies are usually more expensive and are available only for a limited range of antigens. Using microwave treatment and an ABC method, Ichihara *et al* (24) were able to reduce the time for immunostaining frozen sections to 13 min. The most rapid IHC method applied on

frozen sections reported in the literature is the quick LAB method (29). This three-step method is based on 30-sec incubation periods for the primary antibody and detection components.

EnVision uses a dextran polymer coupled to HRP molecules for detection and is a rapid, simple, time-saving and sensitive immunostaining method that can be used to greatly enhance the diagnostic information obtained by standard frozen section examination during surgery. The staining procedure requires no special equipment, and can easily be adapted to different applications in routine diagnostic procedures as well as in research. As a two-step procedure, it can be carried out in a shorter time than the conventional ABC or APAAP methods. There is no need for microwave treatment used in other rapid IHC protocols (23,24,47).

Wiedorn *et al* (48) evaluated the signal amplification power of the Histofine Simple Stain Max (Medac) and compared it to conventional detection methods, Envision+ (DakoCytomation) and GenPoint (DakoCytomation), by testing these systems in 50 paraffin-embedded cervical and portio biopsy specimens for the presence of human papillomavirus by *in situ* hybridisation (ISH). They found that the use of Histofine (combined with AEC+ as chromogen) provided a dramatic increase in signal intensity and sensitivity. Approximately 30% of the samples which were tested negative with conventional detection methods exhibited clear signals when tested with Histofine. In contrast to the signal amplification using biotinyl tyramide (GenPoint), Histofine was easier to use and resulted in less background staining. The signal amplification power of Histofine was comparable to that of EnVision+. Other authors have proven the reliable and sensitive staining results and easy applicability of the Histofine detection system in various types of specimens (49-54). False-positive staining due to endogenous biotin was not observed applying these methods (51) since biotin is not used in the EnVision and Histofine kits.

According to our results, the staining procedures using the EnVision kit/HRP or the Histofine kit can be used in intraoperative frozen section analysis. Antibodies detecting cytokeratin, MUC1 and CD20 showed a comparable immunoreactivity in frozen and paraffin sections applying the EnVision and Histofine kits (55).

In conclusion, the rapid EnVision and Histofine kits investigated in the present study enable immunostaining of frozen sections in less than 13 min. Both procedures represent useful new tools in routine surgical pathology and research, allowing for a more accurate frozen section analysis compared to staining with H&E alone. These techniques can be applied for the classification of poorly differentiated tumors, for the detection of single carcinoma cells in resection margins and for the detection of micrometastases in lymph nodes such as in sentinel nodes.

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