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RESEARCH ARTICLE

Activated macrophages containing tumor marker in colon carcinoma: immunohistochemical proof of a concept

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Abstract The presence of carcinoembryonic antigen (CEA)-containing activated macrophages has been demonstrated in peripheral blood from patients with colorectal carcinoma. Macrophages migrate from the circulation into the tissue, phagocytose debris, and return to the bloodstream. Hence it seems likely that activated macrophages containing tumor debris, i.e., tumor marker, are present in the stroma of colorectal carcinoma. After phagocytosis, they could follow a hematogenic or lymphogenic route to the peripheral blood. The aim of this study is to assess the presence of tumor marker-containing activated macrophages in the stroma of colon carcinoma and in regional lymph nodes. From 10 cases of colon carcinoma, samples of tumor tissue and metastasis-free lymph nodes were cut in serial sections and stained for CD68 to identify macrophages and for CEA, cytokeratin, or M30 presence. Slides were digitalised and visually inspected using two monitors, comparing the CD68 stain to the tumor marker stain to evaluate the presence of tumor marker-positive macrophages. Macrophages containing tumor marker could be identified in tumor stroma and in metastasis-free regional lymph nodes. The distribution varied for the different markers, CEA-positive macrophages being most abundant. The presence of macrophages containing tumor marker in the tumor stroma and lymph nodes from patients with colon carcinoma could be confirmed in this series using serial immunohistochemistry. This finding supports the concept of activated macrophages, after phagocytosing cell debris, being transported or migrating through the lymphatic system. These results support the potential of tumor marker-containing macrophages to serve as a marker for diagnosis and follow-up of colon cancer patients.

Keywords Macrophages · Carcinoembryonic antigen · Tumor marker · Immunohistochemistry · Phagocytosis · Colon carcinoma

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Introduction

Activated (CD14+/CD16+) macrophages containing carcinoembryonic antigen (CEA) and prostate-specific antigen (PSA) have been identified in peripheral blood from respectively colorectal and prostate cancer patients, using flow cytometry [1, 2]. In melanoma-associated macrophages, melanin-containing melanosomes have been described, both at the tumor site as well as in regional lymph nodes [3, 4]. The CD14+/CD16+ subpopulation of monocytes resembles tissue macrophages in many ways [5]. Activated blood-borne monocytes/macrophages are able to migrate into tissues and replenish tissue-specific macrophages, phagocytosing substances (e.g., cell debris) at the site in both the tumor environment as well as in nonneo-

plastic conditions [6–9]. This pilot study intends to evaluate the concept of tumor marker-containing macrophages in colon carcinoma tissue and regional metastasis-free lymph nodes, using double monitor comparison of immunohistochemistry on serial sections to visualize macrophages and tumor markers CEA, cytokeratin, and M30.

Materials and methods

Patient and sample selection

For this pilot study, 10 patients recently operated for colon carcinoma (confirmed on histopathological evaluation) were included. Age, tumor localization, tumor size, and tumor stage according to the American Joint Committee on Cancer (AJCC) were retrieved from medical records. For each case, original histopathological reports and H&E-stained sections were reviewed and the carcinoma sample showing most invasive growth was selected. In one case, the initial diagnostic biopsy was selected for analysis because it showed more invasive growth than the tumor in the colon resection specimen. In one case two separate tumors were found in one resection; we chose to evaluate the more invasive tumor. For each tumor sample, from the same surgical specimen, two regional lymph nodes free of metastasis were selected. All selected samples were evaluated for presence of tumor marker-containing macrophages.

Immunohistochemistry

All paraffin-embedded colon carcinoma samples and lymph node samples were serially sectioned in four consecutive 3-μm slides. Sections were deparaffinized with xylene and rehydrated through graded ethanol. For each sample sections were stained with anti-CD-68, anti-CEA, anti-cytokeratin, and anti-M30 antibodies, respectively.

Antigen retrieval was performed in different ways for the different antigens. For CD68 and M30, specimens were placed in 0.01 M citrate buffer at pH 6.0, incubated for 20 min at 98°C and allowed to cool in buffer. For CEA, specimens were placed in EDTA buffer at pH 9.0, incubated for 20 min at 98°C and allowed to cool in buffer. For cytokeratin, specimens were incubated with 2 mL pepsin 1 mg/mL in 0.1 M HCl at 37°C for 15 min, then cooled and rinsed in tap water. Next, slides were incubated with Dako Dual Endogenous Enzyme Block (Dako) for 5 min to block endogenous peroxidase.

These steps were followed by incubation with primary antibodies. For detection of macrophages, anti-CD-68 (clone PG-M1; Dako Cytomation, Denmark), commonly used as a marker of monocyte–macrophage lineage cells [10–12], was used. To visualize the presence of CEA, clone II-7 was used. This clone is monospecific for CEA [13]. Mouse monoclonal anti-cytokeratin (clone MNF 116, Dako) was used to visualize cytokeratins commonly found in the cytoskeleton of mammalian cells and many carcinomas. Anti-M30 Cytodeath (clone M30; Peviva, Sweden) was used to visualize M30, an antibody that recognizes a neoepitope of cytokeratin 18 exposed after caspase-mediated cleavage during early apoptosis [14]. Antibodies were diluted in Dako Chem Mate Antibody Diluent (Dako). Antibody dilutions used were 1:100 for anti-CD68 and anti-M30, and 1:200 for anti-CEA and anti-cytokeratin.

Antibody binding was visualized using the Dako Autostainer by means of the Dako EnVision Detection System, peroxidase/diaminobenzidine (DAB, Dako) kit for CD68, CEA, and cytokeratin, and aminoethylcarbazole (AEC Single Solution; Zymed, USA) for M30. Slides were incubated with Envision HRP rabbit/mouse solution (Dako) for 30 min and were incubated with DAB (CD68, CEA, and cytokeratin staining) or AEC (M30-staining) for 5 min twice, yielding a respectively brown or red substrate at the site of the target antigen. Finally, all slides were counterstained with haematoxylin and coverslipped.

Scoring of immunohistochemistry

Slides created were digitalized using the 3DH/Zeiss Automatic Brightfield Scan microscopy scanner (Zeiss, Germany) and viewed in MIRAX Viewer (3DHISTECH, Hungary) version 1.12.22.1. Digital slides were evaluated using a double-screen setup, allowing for simultaneous viewing of the CD68 stain on one screen and one of the tumor marker stains from the same case on the other screen. Identical areas were identified, and here, we searched for morphologically macrophage-like cells that stained positively on the CD68 stain and also on the tumor marker stain, illustrated in Fig. 1. Tumor marker-containing macrophages were digitally annotated. Annotations were made in different colors for the respective tumor markers. All slides were evaluated by one observer. In case of doubt, a second independent observer assessed the unresolved cases. If interpretation still remained unclear, the original section was reviewed in conventional microscopy. Ambiguous structures were not marked as positive.

Analysis

Primary tumor The area with the highest density of tumor marker-positive macrophages was identified. In this area, the number of tumor marker-positive macrophages per 2 mm^2 was counted. For each tumor stage and the total group, medians with ranges were determined.

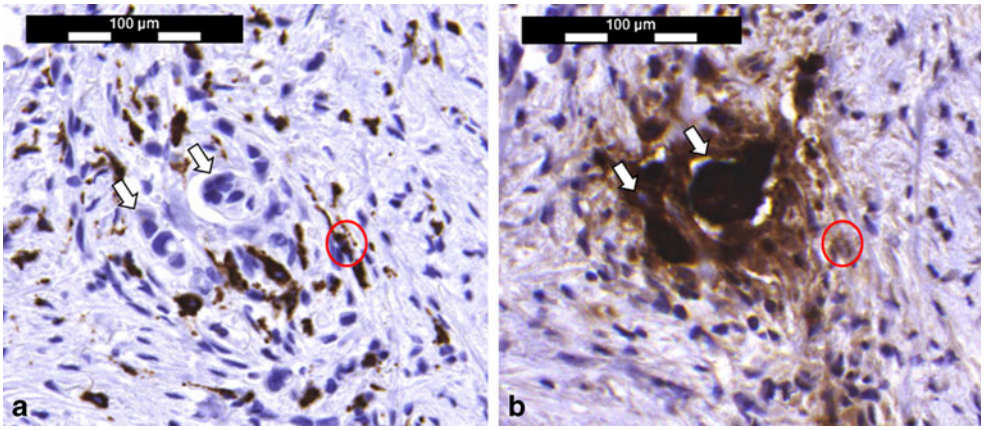

Fig. 1 **a** CD68 stain of tumor sample; **b** corresponding CEA stain of the same sample. Tumor cell clusters are visible (*arrows*) with diffuse staining around tumor cells. An example of a CEA-containing macrophage with granular staining can be seen to the right of the tumor cells (*circle* and *arrowhead*); the CD68 stain shows the presence of macrophages around the tumor and diffusely in the tumor stroma whereas the tumor cell cluster is negative

Lymph nodes The surface area of every lymph node was measured and the number of tumor marker-positive macrophages per 2 mm^2 was recorded for each lymph node. For each tumor stage and the whole group, medians with ranges were determined.

Results

Baseline characteristics

From 10 patients 10 tumors and 20 lymph nodes were analyzed. The median age of patients included in this study was 74 years (range 57–90 years). The male to female ratio was 1:1. Three AJCC stage I tumors were included, one stage II tumor, and six stage III tumors. No stage IV tumors were included in the study. Patient and tumor characteristics are summarized in Table 1.

Digital images

The setup with two monitors, displaying the CD68 and a tumor marker stain, facilitated the examination of the different staining patterns in the same area. By annotating the identified macrophages with different colors for each marker on the CD68 image, differences or similarities in distribution of different markers could readily be identified.

Table 1 Patient and tumor characteristics (n=10)

Gender	Number
Male	5 (50%)
Age	Median in years (range)
Total	73 (57–90)
Male	68 (57–82)
Female	78 (61–90)
Tumor stage (AJCC)	
I (T1–2, N0)	3
II (T3–4, N0)	1
III (any N+)	6
IV (any M+)	0

Tumor stroma

In 10 out of 10 cases, activated macrophages positive for CEA, cytokeratin, and M30 could be identified. Results are summarized in Table 2.

CD68-positive macrophages were encountered in different places. The highest concentrations were found in desmoplastic stroma in the periphery of the tumor. Macrophages were also found directly adjacent to tumor glands, in the lumina of tumor glands, in blood vessels, and in lymphatic vessels. To a lesser extent, macrophages were found in the lamina propria and submucosa of the adjacent normal colon.

A substantial number of activated macrophages showing positivity for CEA could be identified. CEA-positive macrophages were found in all abovementioned localizations of the macrophages. One slide showed a CEA-positive macrophage in a blood vessel wall, suggesting migration of macrophages after phagocytosis (Fig. 2). Most positive activated macrophages showed intracellular deposits of CEA (Fig. 1). In a number of tumors, we observed diffusion of the staining directly adjacent to the tumor epithelium, in an area with a high density of activated

Table 2 Median number of tumor marker positive macrophages in tumor stroma per 2 mm^2 in the area of highest density grouped by AJCC stage, with ranges

Tumor stage (AJCC)	M30	Cytokeratin	CEA
I (n=3)	3 (1–9)	11 (4–21)	362 (93–498)
II (n=1)	2	13	265
III (n=6)	3 (2–10)	10 (4–14)	100 (4–344)
All (n=10) and range	3 (1–10)	11 (4–21)	199 (4–498)

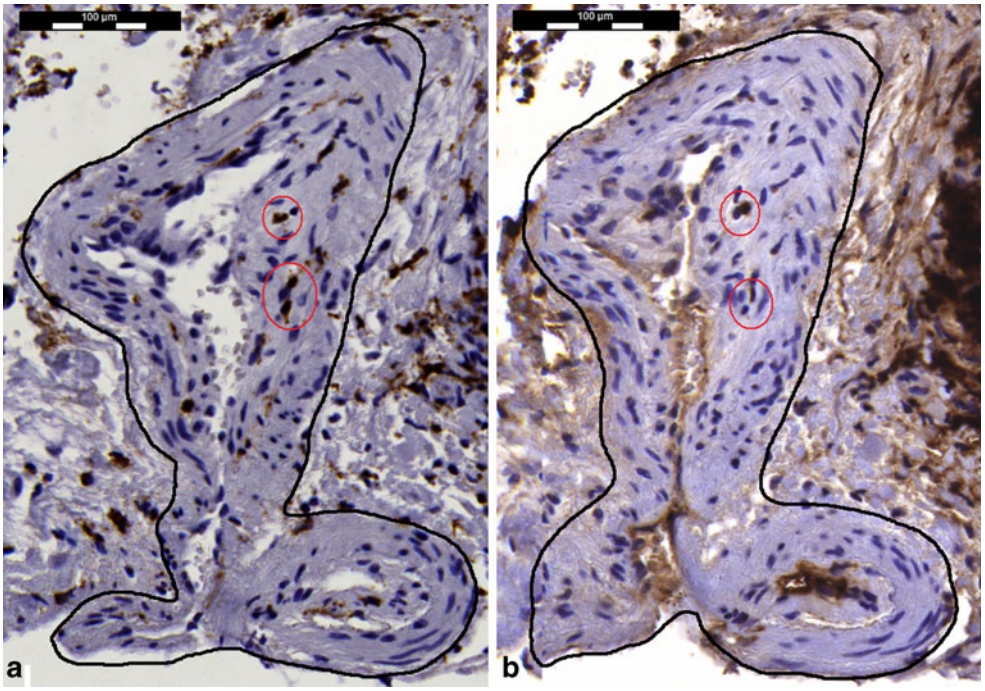

Fig. 2 **a** CD68 stain of a blood vessel (outlined) next to tumor tissue; **b** CEA stain of the same blood vessel. Intramural macrophages are present; two macrophages are positive in the CEA stain (*arrowheads*, *circles*). CEA reactivity is also visible at the luminal borders of this vessel

macrophages (Fig. 1). These macrophages were considered positive. Two stage III tumors were found to be partially CEA negative but still contained a number of CEA-positive macrophages. Cytokeratin-positive macrophages were present in smaller numbers than CEA-positive macrophages (Table 2) and in the serial sections; they were not present at the same location.

M30 tumor cell positivity was found in all tumors, especially within the lumina of tumor glands and in areas of apoptosis. M30-positive activated macrophages were sporadically found in tumor stroma. Since this study assessed properties of tissue-associated macrophages, only these cells were evaluated. The number of macrophages positive for M30 was substantially lower than for CEA and cytokeratin (Table 2). Although this study does not allow any definitive conclusions, the results shown in Table 2 suggest that for higher tumor stages, the number of tumor marker-containing macrophages in the tumor stroma decreases.

Lymph nodes

We evaluated 20 lymph nodes from the same 10 patients. In 19 out 20 lymph nodes, macrophages containing tumor marker could be identified. In one case, M30-containing macrophages could not be identified. Results are summarized in Table 3.

CD68-positive macrophages were found along the lining of the medullary sinuses and in lymphatic nodules. In smaller amounts, macrophages were found in cortical sinuses, medullary chords, and within the cortical parenchyma.

Similar to our findings within the tumor stroma, CEA-containing macrophages were found in relatively large numbers when compared to M30 and cytokeratin. Macrophages with CEA positivity showed a characteristic granular cytoplasmic staining and were almost exclusively found along the lining of the medullary sinuses (Fig. 3).

Cytokeratin-containing macrophages, though fewer in number, showed a less restrictive distribution. These macrophages were found within the sinuses and in the subcapsulary sinuses, medullary chords, and cortex parenchyma as well. Within lymphatic nodules, cytokeratin-containing macrophages were only sporadically seen. Some perivascular elements showed cytokeratin positivity but could be differentiated from macrophages based on morphological characteristics and a lack of corresponding CD68 staining.

Table 3 Median number of tumor marker positive macrophages in lymph nodes per 2 mm^2 grouped by AJCC stage, with ranges

Tumor stage (AJCC)	M30	Cytokeratin	CEA
I (n=6)	0.7 (0.2–1.0)	3.1 (0.3–5.7)	9.5 (1.4–65.7)
II (n=2)	0.6 (0.5–0.7)	5.7 (2.6–5.7)	1.4 (1.4–1.4)
III (n=12)	0.8 (0.0–5.8)	1.4 (0.6–5.9)	3.3 (0.6–53.9)
All (n=20) and range	0.8 (0.0–5.8)	2.3 (0.3–8.7)	4.3 (0.6–65.7)

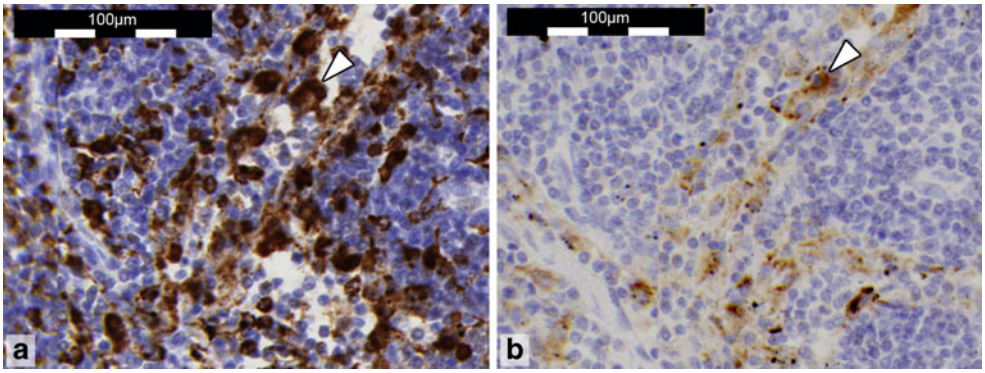

Fig. 3 **a** CD68 stain of a lymph node with widespread macrophage distribution; **b** corresponding CEA stain. CEA-containing macrophages can be seen along the lining of and are limited to the medullary sinuses. Granular staining is visible in a number of macrophages (example at *arrow*)

M30-containing macrophages were found in similar locations as cytokeratin-containing macrophages. In contrast to cytokeratin and CEA, these macrophages were also found in lymphatic nodules. M30-positive macrophages were found only sporadically compared to CEA- and cytokeratin-positive macrophages (Table 3).

Discussion

To the best of our knowledge, this is the first report on the possible route of transportation followed by tumor-associated fragments from the tumor site to the blood stream, supported by morphological and immunohistochemical observations. We propose that circulating tumor marker-containing CD14+/CD16+ macrophages [1, 2] may have ingested this material at the tumor site.

The exact route macrophages follow after phagocytosing tumor material is unclear. In a mouse model of retinal injury, Joly et al. demonstrated the migration of blood-borne macrophages into an area of injury. Microglia, the retinal resident tissue macrophages, showed trafficking abilities towards the injured retinal side and adopted signs of phagocytosis. In reaction to the injury, both at the site of injury and healthy tissue, the number of macrophages rose by influx via blood vessels, adding extra phagocytic capacity to the injured site. Macrophages loaded with debris attaching to and crossing into capillaries suggest a route back into the circulation [15]. Their findings also give support to the hypothesis that tissue-associated macrophages can engulf tumor particles and transport them to vascular structures.

Blood-borne activated macrophages containing tumor material as found in earlier studies [1, 2] have likely obtained tumor material while residing in stromal tissue at the tumor site and afterwards migrated towards the circulation. In this series, macrophages were found mostly at the margin of the tumor and also in the tumor stroma, consistent with earlier findings [16]. We identified a tumor marker-containing macrophage in a blood vessel wall in one section, supporting the hypothesis of hematogenic migration. According to anatomical venous outflow, cells entering the blood vessels will travel through the portal vein towards the liver. Examining (preoperative) portal blood and liver tissue from patients with colon carcinoma could provide more insight into the mechanisms involved in this process.

However, from the assessment of regional lymph nodes, we can conclude that TM-containing macrophages also travel through the lymphatic system towards regional lymph nodes. In case reports of malignant melanoma, melanin-containing macrophages have been demonstrated in sentinel lymph nodes, even without the presence of tumor in the lymph nodes [4, 17]. TM-containing macrophages were more readily identified in lymph nodes than in blood vessels in this study. This may suggest a preference for these cells to travel via lymphogenic route. Although both a lymphogenic and hematogenic route are possible travel itineraries for TM-containing macrophages, our results seem to concur with the observation of metastases being present in lymph nodes more frequent than in organs reached through a hematogenic route such as the liver, the lungs, or the skeletal system.

Macrophages are versatile cells that can exhibit different functional phenotypes. Macrophages have been divided into the M1 and M2 type. M1 macrophage activity includes proinflammatory functions, including the capacity to kill microorganisms and the production of proinflammatory cytokines. M2 macrophages are involved in immunosuppression, tissue remodeling, angiogenesis, and scavenging of debris, including apoptotic cells [18, 19]. The division in M1 and M2-type macrophages may not be absolute [20], enabling macrophages to switch from one function type to another or assuming characteristics of more than one type at a time. Tumor-associated macrophages may play an important role in tumor progression, angiogenesis, metastatic ability, and remodeling of the extracellular matrix [21].

Wyckoff et al. [16] found that macrophages only phagocytosed labeled sugars while in the stroma of a tumor, as opposed to while circulating in the blood. This may indicate a phenotype difference between blood-borne

and tissue-associated macrophages, possibly under influence of cytokines and (tumor-derived) growth factors [22–24]. Nuclear condensation as seen in blood-borne tumor marker-containing macrophages [1] could indicate macrophages may have reached the end of their life cycle and initiate apoptosis after fulfilling their role in tumor stroma. A vitality stain could demonstrate whether blood-borne macrophages containing tumor material are indeed in the process of apoptosis.

Tumor marker containing in macrophages showed a typical granular staining pattern. In 2010, Lazova et al. [3] described melanoma-associated macrophages containing coarse melanin in a similar staining pattern. Electron microscopy and immunohistochemistry showed that these melanosomes were contained in autophagosomes and/or autolysosomes in the macrophage cytoplasm. This may also be the case with CEA in colon carcinoma-associated macrophages.

Pawelek et al. [25] have postulated the fusion of tumor cells with bone marrow-derived cells as a possible explanation for tumor progression and metastasis. Such hybrid cells have been identified in vitro and in sporadic cases in vivo. The presence of tumor marker in macrophages could be a characteristic of tumor–macrophage hybrid cells. Electron microscopy may be of use to further evaluate the interaction between macrophages and tumor material.

We found substantial differences between the various tumor markers in the densities of tumor marker-containing macrophages. This gives rise to a number of hypotheses. Firstly, there may be a relation between the presence and distribution of the investigated substances in the tumor and the possibility to find loaded macrophages. Rupa et al. described the amount of M30-positive cells in colorectal adenocarcinomas to be 5–17%, with a mean of 11.1% [26], while almost all colorectal carcinoma epithelial cells exhibit cytokeratin and CEA positivity. Therefore, the argument of varying presence of tumor marker might be true for CEA compared to M30 but is not likely to be true for CEA compared to cytokeratins. Secondly, the stability of the substance may play a role. CEA is an extremely stable molecule that can even survive perchloric extraction, while cytokeratins are easily damaged by electrical or chemical agents. Thirdly it might be that the high level of glycosylation of CEA facilitates the interaction with tissue macrophages compared to cytokeratins. Fourthly, a macrophage does not only phagocytose substances. Phagosomes fuse with lysosomes containing various enzymes, which may degrade antigens, making them unrecognizable to our antibodies. A combination of all the abovementioned mechanisms—availability, selective phagocytosis, molecule stability, and degradation—is also possible.

Although we did not apply double staining procedures, the serial section approach consistently showed different areas of distribution for macrophages with the three antigens investigated in this pilot study. This was observed both in the tumor stroma but even more prominent in the lymph nodes. Cross-reaction with the nonspecific cross-reacting antigen, a macrophage component, might make interpretation more difficult; however, clone II-7 was one of the highly CEA-specific antibodies in a comparative study on multiple CEA antibodies [13]. Also, the fact that CEA-positive macrophages were not found in all lymph nodes and their distribution in the lymph node was limited to a subset of cells gave further support to a meaningful interpretation of this observation.

The use of double chromogenic stains does not seem to be an alternative because we attempt to stain different structures both located in the cytoplasm of a single cell. Using direct fluorochrome-labeled antibodies and confocal laser scanning microscopy may be a better option to answer the question of colocalization of different substances in the same macrophages. Considering the typical granular staining of intra-macrophage tumor marker, the observed anatomical distribution of tumor markers and macrophages, and morphological characteristics of macrophages, serial section immunohistochemistry seems adequate for this pilot experiment evaluating whether or not tumor marker-containing macrophages are present in the colorectal tumor stroma and lymph nodes.

Conclusion

In this study we were able to identify tissue-associated macrophages both in tumors and in their lymphatic draining area. Serial sectioning and immunohistochemistry are relatively simple methods to identify tumor marker-containing macrophages.

Macrophages containing tumor material are present in the stroma of colon carcinoma and in regional metastasis-free lymph nodes. The number of tumor marker-positive macrophages varied between cases as well as between the different tumor markers. The distribution of tumor marker-positive macrophages is different for the various markers used in this study.

Conflicts of interest None.

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