Myofibroblasts Correlate with Lymphatic Microvessel Density and Lymph Node Metastasis in Early-stage Invasive Colorectal Carcinoma

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Abstract. Background: Recent studies have shown that the interactions between tumor cells and stromal cells are important in tumor development. A possible correlation between tumor-activated myofibroblasts, the main component cells of tumor stroma, and lymphatic microvessel density (LMVD) or other clinical parameters in carcinoma was investigated. Materials and Methods: Immunohistochemical examination of alpha-smooth muscle actin and podoplanin were performed in 83 cases of early-stage invasive colorectal carcinoma. Results: There was a good correlation between proliferation of myofibroblasts (PMpt) and LMVD (LMVDpt) in the peri-tumoral area (p=0.0034). Increased PMpt was also associated with lymphatic invasion (p=0.0051) and with lymph node metastasis (p=0.011). However, proliferation of myofibroblasts in intra-tumoral (PMit) areas was not associated with these clinical parameters. Conclusion: Proliferation of myofibroblasts in peri-tumoral areas seem to play an important role in lymphangiogenesis, and is also associated with lymph node metastasis.

The proliferation, invasion and metastasis of tumor cells require complex interactions between the tumor cells and the surrounding matrix. Recent studies have shown that the interaction between tumor cells and stromal cells in different organs is important in the development, differentiation and proliferation of tumor cells (1-4).

Myofibroblasts are the main component cells in tumor stroma, and alpha-smooth muscle actin (α-SMA)-positive myofibroblasts were found to participate in the synthesis of extracellular matrix components of tumor stroma, and to produce lytic enzymes able to degrade the basement membrane surrounding tumor glands. Although present in the progressive tumor nodules, they disappear during tumor regression (5, 6). The correlation between microvessel density and myofibroblasts was shown by Zidar et al. (7), but the influence of myofibroblasts in lymphangiogenesis remains unclear. Only recently, podoplanin, a 43-kd glomerular podocyte membrane mucoprotein and a specific lymphatic vessel marker, has enabled the investigation of the lymphatic microvessel density (LMVD) in paraffin-embedded sections (8, 9).

In this study, the correlation between the proliferation of α-SMA-positive myofibroblasts (PM) and LMVD, as well as other clinical parameters in early-stage invasive colorectal carcinomas, were investigated.

Materials and Methods

Patients and tissues. Eighty-three patients with early-stage invasive colorectal carcinoma, defined as carcinoma that invades only the submucosal layer of the bowel wall (10, 11), who underwent resection from 1985 to 2004 in our department, were studied. The group consisted of 57 men and 26 women, with a mean age 67.5 years (range 39~88). Of these, 64 tumors were in the colon and 19 in the rectum. The macroscopic and histological findings including gross type, depth of tumor invasion, histology, lymphatic invasion and lymph node metastasis were evaluated based on the criteria of the Japanese Research Society for colorectal cancer (12). The depth of submucosal invasion was divided into three grades: (i) Sm1, carcinoma invading into the upper one-third, thus showing minimum submucosal invasion; (ii) Sm2, carcinoma invading into the middle part of the submucosa; (iii) Sm3, carcinoma invading into the lower layer of the...
submucosa (13). Of these 83 patients, 7 (8.4%) patients had lymph node metastasis.

Immunohistochemistry. Immunostaining for α-SMA and podoplanin was performed using the Envision+/HRP method with heat-induced antigen retrieval. One or two representative blocks of each tumor were selected for immunostaining analysis. Serial 3-μm paraffin sections containing the tumor margin were dewaxed in xylene, rehydrated in alcohol, and for staining podoplanin the sections were heated to 95°C in an oven (650W) for 45 min to reactivate the antigen. Endogenous peroxidase activity was suppressed by a solution of 3% hydrogen peroxide in methanol for 20 min. After being rinsed three times in phosphate-buffered saline (PBS), sections were incubated for 90 min at room temperature with monoclonal antibody against α-SMA (1A4, DAKO, Glostrup, Denmark), or monoclonal antibody against podoplanin (11-003, AngioBio, Dal Mar, CA, USA). After washing in PBS, the sections were treated with goat anti-mouse immunoglobulins conjugated to peroxidase-labelled-dextran polymer (Dako, Carpinteria, CA, USA) for 1 h at room temperature. Then the sections were washed in PBS and developed in 0.05 M Tris-HCl buffer (PH=7.5) containing 0.6 mg/ml 3,3’-diaminobenzidine tetrahydrochloride (DAB) for 4 min for myofibroblast staining and 10 min for podoplanin staining at room temperature. After washing in water, the nuclei were counterstained with Mayer’s hematoxylin. Negative control sections were stained by omitting the primary antibody.

Tumor size was measured by minor axis x major axis (mm²) on the hematoxylin and eosin specimen. Determination of PM was performed according to the Quickscore method (14). Briefly, the staining intensity was scored on a scale of 3 (1=weak, 2=moderate, 3=strong), and the proportion of the stroma in or adjacent to the tumor staining positively was scored out of 4 (1<25%, 2=25-50%, 3=51-75%, 4=76-100%). The score for intensity was added to the score for proportion to give a score in the range of 0-7 and grouped as – (score=0), + (score=1-3), ++ (score=4-5), +++ (score=6-7). Degrees of both intra-tumoral and peri-tumoral PM were scored on each section.

Determination of LMVD was performed as suggested by Weidner et al. and Straume et al. (15, 16). Briefly, the sections were scanned at low magnifications (x40 and x100) to identify the tumor areas with the highest amount of lymphatic vessels (“hot spots”). Within these areas, five fields at x200 magnification (high power field (HPF), 0.708 mm²/field) were examined, and the mean value of these fields were calculated. Vessels more than one-half HPF (x200) away from the invasive front, were not counted. Any endothelial cell or cell cluster, highlighted by podoplanin reactivity and clearly separate from adjacent vessels, tumor cells and connective tissue elements, was regarded as a distinct countable vessel (14). Special LMVD counts were established for LMVD in peri-tumoral (LMVDpt) areas for each case, and the counts are given as vessels per millimeter squared. The predominant appearance of peri-tumoral lymphatic vessels was also recorded (compressed and/or angulated versus rounded).

In all 83 colorectal carcinomas, sections stained for α-SMA and podoplanin were scored independently by Pin Liang and J. W Hong.

Statistics. The Kruskal-Wallis test, Mann-Whitney test, Spearman test and logistic regression were used as appropriate. For all tests, a two-sided value of 0.05 or less was considered statistically significant.

Results

Myofibroblasts appeared as α-SMA-positive spindle-shaped stromal cells with long cytoplasmic extensions. Their distribution in normal mucosa was scarce (Figure 1), with periglandular myofibroblasts forming a layer around the glandular epithelium, as described by Hewitt et al. (17). In carcinomas, myofibroblasts were very much more abundant within the tumor stroma, in moderate to high abundance, and were stained more intensively in the intra-tumoral zone than in the peri-tumoral zone (Figure 2). The PMit was identified in all 83 patients, while the PMpt was identified in 68 cases (82%). The Kruskal-Wallis test revealed a significant difference in LMVDpt in cases with different degrees of PMpt (Figures 3, 4 and Table I). Subsequent Mann-Whitney U-tests revealed a significant difference in LMVDpt between cases with score+ and score+++ (p<0.0001) and between cases with overall score+ and score+++ (p<0.0001). No significant difference in LMVDpt between cases with overall score+ and score++ was observed (p=0.203). The PMpt was also significantly associated with lymphatic invasion and lymph node metastasis (Figure 5 and Table I). In addition, multivariate analysis of different effects, presumably associated with lymph node metastasis, is shown in Table II. As a result, the degree of submucosal invasion lymphatic invasion and of PMpt emerged as independent predictors of lymph node metastasis. The degree of PMit was positively related to that of PMpt (p<0.001 Spearman test), but no significant correlation was found between PMit and different clinical parameters (Table I).

As shown in Figures 4 (B) and 5, lymphatic capillaries were identified as weakly stained, inconspicuous vessels when stained with anti-podoplanin antibody. LMVDit was rare, whereas the majority of intra-tumor hot spots were most frequently found in close proximity to the tumor border, as reported by Peter et al. (18). Evaluation of the LMVDit gave a median count of 2.1 per millimeter (range 1.0 to 9), while the median count of LMVDpt was 7.3 (range 2.0 to 22). The difference was significant between LMVDpt and LMVDit (p<0.01 Mann-Whitney test).

Discussion

We set out to determine whether α-SMA-positive PM is associated with LMVD as well as with other clinical parameters in early-stage invasive colorectal carcinomas. Myofibroblasts have heterogeneous cytoskeletal phenotypes with regard to their content of intermediate filaments (vimentin and desmin), α-SMA, β actin, γ actin and myosin (19). Our study focused only on α-SMA-positive stromal cells. In colorectal carcinoma, myofibroblasts have been reported to proliferate at the invasive front, thereby altering the adhesive
Figure 1. Myofibroblasts detected by α-smooth muscle actin (α-SMA). There is generally little evidence of myofibroblasts in the connective tissues of normal mucosa. Original magnification x100.

Figure 2. Myofibroblasts detected by α-smooth muscle actin (α-SMA), are abundant and stained more intensively in the intra-tumor zone than in the peritumoral zone. Original magnification x100.
Figure 3. Distribution of low proliferation peri-tumoral myofibroblasts accompanied with low lymphatic microvessel density (LMVD). α-Smooth muscle actin (A) and podoplanin (B) detection on serial sections of early-stage of colon carcinoma. Original magnification x200.
Figure 4. Distribution of highly proliferating myofibroblasts (PMpt) accompanied with high lymphatic microvessel density (LMVDit). α-Smooth muscle actin (A) and podoplanin (B) detected in serial sections of early-stage of colon carcinoma. Original magnification x200.
and migratory properties of colorectal carcinoma cells (5, 20). Myofibroblasts were more abundant within the tumor stroma than in normal tissues; furthermore they did not show a uniform distribution. This is the first study where tumor-activated PMpt was found to be significantly associated with LMVDpt as well as with lymphatic invasion and lymph node metastasis in early-stage invasive colorectal carcinoma.

Some earlier studies have proven that cancer cells and stromal cells could promote the secretion of pro-angiogenic and anti-angiogenic molecules in human carcinoma. These pro-angiogenic molecules such as VEGF and VEGF-c secreted by tumor cells are believed to be the main factors inducing blood and lymph vessel neoangiogenesis (21, 22). This study has revealed that myofibroblasts in peri-tumoral areas also play an important role in lymphangiogenesis, at least in early stage invasive colorectal carcinoma.

Our analysis showed no association between PMit and LMVDit (p=0.812 Kruskal-Wallis test). Furthermore, the score of PMit was higher than that of PMpt, whereas that of LMVDit was less than that of LMVDpt. These data could be explained by the fact that the resistance of lymphatic vessels, to pressure is lower than that of blood vessels which possess smooth muscle cells, thus rendering it difficult for lymphatic vessels to retain their pattern in the center of tumors. According to Curti et al. (23), interstitial pressure is significantly higher within the tumor than outside. The average size (cross-sectional area) of the lymphatic vessels in the center of tumors was found to be less than that in peri-tumoral areas. LMVDpt is greater than LMVDit, and the fact that intra-tumor hot spots were most frequently present in close proximity to the tumor border also support our speculation. Padera et al. have also found that, although there were fewer lymphatic vessels in intra-tumoral areas, they were not functional because of the higher pressure (24).

The difference between PMpt and PMit with regard to maturity and origin could also be responsible for their different functions. The function of tumor-activated myofibroblasts around the tumor cells differs with the passage of time. Recently, Bourreyron et al. have shown that myofibroblasts in culture before the fifth passage induce LoVoC5 colon cancer cell proliferation, spread and adhesion. After the 15th passage, myofibroblasts have been found to lose these functions (25). Studies have also shown that the origin of myofibroblasts at the invasive edge of a tumor and within tumors is different in early stage colorectal carcinomas, and that myofibroblast maturity is
also different in different types of gastric carcinomas (26, 27). Our results revealed that the staining intensity of PMit was significantly higher than that of PMpt. Differences in maturity and origin may also lead to different PMpt and PMit functions in lymphangiogenesis. Further studies of the mechanism of function in lymphangiogenesis are required.

Our findings also indicate that the degree of PMpt, but not of PMit, was closely linked to aggressive biological behavior, including lymphatic invasion and lymph node metastasis. Moreover, multivariate analysis demonstrated the degree of PMpt to be an independent predictor of lymph node metastasis. It has been emphasized that myofibroblasts have a role in the up-regulation of production of the MMP-2 and U-PA enzymes, which have been proven to be connected with tumor invasion and metastasis (5, 28, 29). Furthermore, the myofibroblast cells both in the intra-tumoral and peri-tumoral areas may prevent physical contact between tumor cells and immune cells, which is an essential phenomenon for the effective destruction of tumor cells (30). These results point to tumor cells defined by a higher degree of PMpt and LMVDpt in peri-tumoral areas, and more likely to invade adjacent normal and lymphatic tissue.

In conclusion, the results of our study indicate that myofibroblasts in peri-tumoral areas seem to play a role in peri-tumoral lymphangiogenesis, and that the degree of proliferation of myofibroblasts in peri-tumoral areas is a new and strong predictor of lymph node metastasis. Further studies dealing with peri-tumoral myofibroblasts may suggest new therapeutic strategies to limit tumor spread.

### Table I. Correlation between proliferation of myofibroblast (PM) and clinical parameters.

<table>
<thead>
<tr>
<th></th>
<th>Intra-tumoral PM</th>
<th>Peri-tumoral PM</th>
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<tr>
<td></td>
<td>+</td>
<td>++</td>
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<tr>
<td>Macroscopic type&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Elevated</td>
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<tr>
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<td></td>
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<tr>
<td></td>
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<td>4</td>
</tr>
<tr>
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<td>sm3</td>
<td>7</td>
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<td>9</td>
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<tr>
<td></td>
<td>21 mm or greater</td>
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<td></td>
<td>negative</td>
<td>15</td>
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<td>LMVD (median±SD/mm&lt;sup&gt;2&lt;/sup&gt;)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.4±4.3</td>
<td>6.8±3.3</td>
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<sup>a</sup>Kruskal-Wallis test
<sup>b</sup>Mann-Whitney test

### Table II. Logistic regression analysis in relation to lymph node metastasis.

<table>
<thead>
<tr>
<th></th>
<th>Coefficient of regression</th>
<th>χ&lt;sup&gt;2&lt;/sup&gt;</th>
<th>P-value</th>
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<td>moderately-differentiated</td>
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<td>Wall invasion</td>
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<td>sm1 or sm2 vs. sm3</td>
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<td>Lymphatic invasion</td>
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<td>LMVD</td>
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<tr>
<td>PMpt</td>
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<td>4.381</td>
<td>0.036</td>
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References


