Liver-metastatic Potential of Colorectal Cancer Is Related to the Stromal Composition of the Tumour

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Abstract. Background: The tumour stroma is an important modulator of cancer cell behaviour. The aim of this study was to compare the stromal composition between primary colorectal cancer (CRC) and colorectal liver metastases (CLM). Materials and Methods: The stromal composition in matched tissue sections of CRC and subsequent CLM was analysed, and related to clinical parameters. Results: Differences in the expression pattern of type I collagen and type IV collagen in CRC was related to a higher risk of CLM. Two types of CLM the desmoplastic and pushing type were identified. The time between CRC and diagnosis of CLM was shorter (p=0.047) for desmoplastic CLM. The mortality rate was higher for pushing CLM due to frequent extrahepatic disseminated disease. A difference in the overall survival rate between patients with desmoplastic and those with pushing CLM was seen at follow-up of more than 60 months (p=0.046). Conclusion: The liver-metastasizing potential is related to the stromal composition of primary CRC. There are distinct growth patterns in CLM with differences in stromal composition and clinical outcome.

Despite intensive research, better diagnostic opportunities, and improved treatment options, colorectal cancer (CRC) remains one of the leading causes of cancer-related mortality globally (1). The major cause for mortality in CRC is the metastatic spread of the disease, with approximately 50% of patients with CRC developing liver metastases (CLM). We previously reported on a distinct up-regulation of type IV collagen in CLM tissue, and on elevated circulating levels of type IV collagen fragments in patients with CLM, but not in patients with primary CRC (TNM stage I-III) (2). Additionally, the levels of circulating type IV collagen reflect the hepatic tumour burden, suggesting that circulating type IV collagen could be used as a tumour marker (2). Taken together, these findings indicate that type IV collagen has a role in CLM.

A tumour consists of cancer cells and tumour stroma. The stroma is composed of extracellular matrix (ECM) components, such as different collagens, fibronectin, proteoglycans, and glycosaminoglycans, and by the non-malignant cells of the tumour, such as endothelial cells, fibroblasts, and immune cells. Collagens are proteins found in both normal and tumorous stroma. In normal intestine, type I and III collagens are found in the interstitial compartment, and type IV collagen in the epithelial and endothelial basement membranes (BM) (3). In normal liver, type IV collagen is located in the liver sinusoids and the vascular BMs (4). Type I collagen is mainly located in the portal tracts, and type III collagen is found sparsely in the liver sinusoids and portal tracts (5). Previously, the stroma in primary CRC was regarded as a reactive inflammatory and sometimes as a part of the protective response against invading cancer cells (6), and was not considered to support tumour progression. During the past decades, however, the importance of the stroma in providing survival cues for cancer cells has become increasingly evident (7-10). Whether there are differences between the stroma of a primary cancer and that of distant metastases arising from that tumour is unknown. In addition, it is not known if the stroma of metastasizing cancer differs from that of cancer that does not metastasize.

The growth pattern of tumour stroma in CLM, in relation to inflammation and angiogenesis, has been characterized. Two dominant growth patterns, the desmoplastic and the pushing type of CLM were found (11). In the desmoplastic growth pattern, the cancer cells and the normal liver tissue are separated by a thick desmoplastic rim, with a rich inflammatory infiltrate and increased angiogenesis. In the pushing growth pattern, cancer cells push the normal liver aside, and only a small desmoplastic reaction separates the...
tumour and the normal liver tissue, with mild inflammation and less angiogenesis. A more unusual, replacement growth pattern was also described, where cancer cells grew next to the hepatocytes in normal liver architecture, with little or no inflammation or angiogenesis present.

Our aim was to study whether the stromal composition in CRC differs between non-metastatic CRC and liver-metastatic CRC. Additionally, we sought to verify the previously described different growth patterns of CLM, and analyse if it is possible to predict the growth pattern of the CLM based on stromal characteristics of the primary tumour, and whether this translates into differences in the clinical outcome of the disease.

**Materials and Methods**

**Patient cohort.** Forty-eight patients who underwent surgery for both primary CRC and CLM in Västerbotten County, Sweden between 1998-2009 were included in this study. The group was selected on the basis that tissue samples were available from both the primary CRC and the corresponding CLM. A group of 31 patients who underwent surgery for primary CRC without clinical or radiological evidence of metastatic disease, or local recurrence for at least five years postoperatively, served as a control group. The postoperative follow-up was carried out according to protocols with clinical and radiological follow-up every 3-6 months for two years and then every 6-12 months up to five years. The groups were age- and gender-matched (Table I). Clinical parameters (TNM stage, differentiation grade of CRC, primary CRC site, Carcinoembryonic antigen (CEA) level at the time of CLM, size of the largest CLM, number of CLM, oncological treatment, recurrence of CLM, and time interval between CRC to detection of CLM) were collected from the patients’ charts. Several of the patients with rectal cancer in both groups had received neoadjuvant radiation therapy (CLM group n=10 and controls n=9), and in three cases also chemotherapy for down-staging. Additionally, 16 patients had received adjuvant or neoadjuvant chemotherapy before surgery of the CLM. This study was approved by the ethical committee (EPN) of Northern Sweden, and informed consent was obtained from all patients. Patient characteristics are shown in Table I.

**Tissue samples and histological grading.** Paraffin tissue blocks from the primary CRC and the subsequent CLM from the same patient (n=48) were obtained from the Department of Pathology at Umea University Hospital. For the control group, paraffin tissue blocks from the CRC specimens (n=31) were collected. For immunohistochemical analysis of the primary CRC, tissue blocks containing the most invasive front of the tumour were used. Sections of 5 μm were cut using a microtome (Leica RM 2165, Leica Microsystems, Wetzlar, Germany). The expression intensity and the patterns of stromal collagens were graded as 0=no expression, 1=mild to moderate expression, and 2=strong expression. The expression intensity in CRC was graded both in the desmoplastic reaction (DR) and in the immediate vicinity of the cancer cell. For the analysis of the growth pattern of CLMs, one tissue block containing a representative fraction of the tumour-liver parenchyma interface per patient was used. In patients with multiple CLM, the largest metastasis was used. When classifying the growth pattern of the metastasis, only the dominant pattern was used for classification according to previous description (11). The expression intensity and pattern of stromal collagens in CLM was graded as 0=no expression, 1=mild to moderate expression, and 2=strong expression. The expression of collagens in CLM was graded both at the tumour border and within the tumour. The histological grading of the collagen expression in both CRC and CLM was related to clinical parameters. For microscopy and image capture, a Nikon Eclipse E 600 microscope, Nikon DXM1200 digital camera and NIS elements F 2.30 software were used (Nikon Systems Inc., Tokyo, Japan). The immunohistochemical analysis was performed by H.N.
Immunohistochemical and chemical staining. Routine staining was performed with haematoxylin and eosin. Chemical reticular staining was used for the visualization of reticular fibers. The Picro-Sirius Red (Polysciences, Inc. Warrington, PA, USA) staining kit was used for staining of type I and II collagens. This stain differentiates between type I and III collagen fibers using polarized light. All immunohistochemical staining was performed using the Ventana Benchmark automated immunostainer (Ventana Medical Systems, Tucson, AZ, USA). The primary antibodies, pre-treatments, and concentrations used, were anti-human type I collagen (EDTA, rabbit polyclonal, no. 34710 1:1200 incubated for 32 min at 37°C; Abcam, Cambridge, UK) and anti-human type IV collagen (protease, rabbit polyclonal, MP Cappel 1:75, incubated for 32 min at 37°C; Fischer Scientific, Gothenburg, Sweden). A secondary antibody linked to peroxidase, followed by diaminobenzidine tetrahydrochloride (DAB) as a chromogen, was used.

Statistical analysis. SPSS version 19 was used for statistical analysis. Differences between groups were analyzed using odds ratio (OR) and the Pearson's chi-square test. Mann-Whitney U-test was used to estimate the differences between disease-free time intervals. Probability of survival was estimated using the Kaplan-Meier estimator, and differences were analysed with the log-rank test.

Results

High collagen expression in liver-metastasizing primary CRC. Liver-metastasizing primary CRC differed significantly in collagen expression compared to the non-metastatic control group (Figure 1). CRC that metastasized to the liver displayed a significantly higher expression of type I collagen in the DR (p=0.002) (Figure 1). Additionally, type IV collagen was expressed intensely in the vicinity of the cancer cells, in liver-metastatic CRC when compared to non-metastasizing CRC (p=0.004) (Figures 1 and 2), but only at very low levels in the DR, in both the non-metastatic and in the liver-metastatic CRC. Patients that had received preoperative radiotherapy for rectal cancer displayed a higher collagen expression, in general, in both the control group (n=9) and in the CLM group (n=10).

The presence of high collagen I expression levels in the vicinity of cancer cells and in the DR correlated to an increased risk of being diagnosed with a subsequent CLM (OR=3.616, [95% CI=1.604-12.286, and OR=6.00 [95% CI=2.096-17.173, respectively). The presence of high levels of reticular fibers was also associated with an increased risk of being diagnosed with CLM (OR=3.419, [95% CI=1.161-10.123). However, the highest risk of developing a subsequent CLM was found in patients with strong type IV collagen expression in the immediate vicinity of the CRC cells (OR=11.3, [95% CI=3.706-34.726). The expression of type I collagen in the DR of primary CRC and the expression of type IV collagen in the vicinity of the cancer cells correlated to a high differentiation of the CRC (p=0.019 and p=0.052, respectively) (Table II). No correlation between the expressions of collagens in relation to T-stage of the CRC was observed (Table II).

Table II. Type I and IV collagen expression in liver metastatic colorectal cancer correlated to T-stage and tumour differentiation.

<table>
<thead>
<tr>
<th>Primary CRC expression</th>
<th>T Stage</th>
<th>Differentiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen I TC</td>
<td>0.414</td>
<td>0.690</td>
</tr>
<tr>
<td>Collagen I DR</td>
<td>0.575</td>
<td>0.019</td>
</tr>
<tr>
<td>Collagen IV TC</td>
<td>0.659</td>
<td>0.052</td>
</tr>
<tr>
<td>Collagen IV DR</td>
<td>0.609</td>
<td>0.451</td>
</tr>
</tbody>
</table>

TC, Expression in the vicinity of the tumour cells; DR, expression in the desmoplastic reaction.

High expression of type IV collagen in the vicinity of invading cancer cells in CRC. Type IV collagen was seen to be highly expressed in close contact with the cancer cells of the liver-metastatic CRC, but not so pronounced in the surrounding DR (Figure 2). This differed from non-metastatic CRC, which only had a weak type IV collagen expression in general. In CRC with subsequent CLM, a rim of intense type IV collagen expression surrounding the islands of cancer cells invading the deeper layers of the intestinal wall was observed (Figure 2), and this higher expression differed significantly from that in the control group (p=0.004). Type I collagen in metastasizing CRC, was mainly expressed in the DR and this differed significantly from the control group (p=0.002), but it was also expressed to an extent in the vicinity of the CRC (Figure 2), although not as strongly as type IV collagen.

Two distinct growth patterns identified in CLM. Two growth patterns of CLM were identified (Figure 3): a group with a desmoplastic growth pattern (n=22), characterized by the presence of a thick DR, rich in collagen surrounding the CLM and an intense inflammatory infiltrate; and a second group with the pushing growth pattern (n=25), where the tumour cells were separated from the normal liver parenchyma by merely a thin rim of collagen and with only a mild inflammatory infiltrate present. In most of the CLM samples, only one growth pattern was present. In a few cases where both growth patterns were present, the dominant pattern was used for analysis, as previously described (11). Five patients underwent additional surgical resection due to recurrent disease in the liver. Most interestingly, the growth pattern and collagen expression in these metastases were always the same as in the first CLM. CLM with a desmoplastic growth pattern displayed significantly higher levels of type I and IV collagens in the DR surrounding the tumours, when compared to the pushing group (p<0.001 and p<0.001, respectively).
Figure 1. Expression patterns of collagens in normal colon (A, D, G,), non-metastatic colorectal cancer (non-met CRC) (B, E, H) and liver-metastatic colorectal cancer (liver-met CRC) (C, F, I). H&E is haematoxylin-eosin stain; COL-I visualizes type I collagen (brown). COL-IV visualizes type IV collagen (brown). Cancer cells are indicated by “T”. Type I collagen was found to a greater extent in the desmoplastic reaction (DR) (arrows) of the tumour in metastatic CRC when compared to non-metastatic CRC (E, F). Type IV collagen was predominantly found in close contact with the cancer cells (arrow) in metastatic CRC (I) compared to non-metastatic CRC, which displayed low levels of type IV collagen in general (K). Histological analysis shows significantly higher type I collagen levels in the DR of metastatic CRC compared to non-metastatic CRC (**p=0.002) (J), and significantly higher type IV collagen expression in the vicinity of the cancer cells (TC) in metastatic CRC (**p=0.004) (K). Data for staining intensity and pattern are presented as the mean±SD (non-met CRC n=31 and met CRC n=48, respectively).
Out of four patients who had received neoadjuvant chemotherapy, all responded to the treatment with stable disease or partial response of their CLM (one and three patients with the desmoplastic and pushing type of CLM, respectively). In the tissue analysis, regression with empty spatia and tumour cell necrosis was seen. However, despite chemotherapy, the metastatic pattern in these patients could still be easily determined.

**Shorter time interval between primary CRC and CLM in the desmoplastic group.** The desmoplastic group had a significantly shorter ($p=0.047$) time interval between primary CRC and detection of CLM (median=0.0 [range=0-41] months) compared to the pushing group (median=7.0 [range=0-52] months). The CEA level in the pushing group (median=67.5 [range=0.8-267] ng/ml) was higher at the time of diagnosis of CLM, when compared to that for the desmoplastic group (median=5.1 [range=0.8-1712] ng/ml). However, the difference was not statistically significant due to the extreme variations of the CEA levels within the groups. No relationship between the stromal composition of the primary CRC, and the subsequent metastatic pattern of the CLM arising from that tumour was observed. Therefore, the metastatic pattern in the CLM could not be predicted by analyzing the stroma in the primary CRC. Additionally, there was no relation between the growth pattern of CLM and gender ($p=0.654$), T-stage ($p=0.467$), differentiation of the CRC ($p=0.187$), primary CRC localization ($p=0.850$), size of the largest CLM ($p=0.122$), or number of CLM ($p=0.190$).

**Shorter overall survival in patients with the pushing type of CLM.** Patients in the pushing group had a shorter overall mean survival after liver resection (63±6.9 months) than patients with the desmoplastic growth pattern in the CLM (93±10.5 months), a difference reaching borderline significance ($p=0.100$) (Figure 4) From the survival curves, it is also obvious that there were more long-term survivors in the desmoplastic group; furthermore, when analysing the overall survival in patients at a follow-up time interval of more than 60 months ($n=19$), a significant difference in the overall survival was seen ($p=0.046$). For patients with a follow-up of less than 60 months ($n=26$), no statistical difference was seen.
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Normal liver  Pushing  Desmoplastic

HE x10

D

RET x10

G

COL-I x10

J

COL-IV x10

M

N

Mean COL-I expression

Mean COL-IV expression

*
Pushing growth pattern of the CLM is related to risk of lung metastases. The causes of death were characterized for the whole cohort. Two patients died of causes other than cancer and were excluded from this analysis. Interestingly, patients in the pushing group developed extrahepatic metastatic disease (Table III) more frequently. More specifically, nine of the patients in the pushing group developed lung metastases, compared to none in the desmoplastic group. The majority of the deceased patients in the desmoplastic group had recurrence restricted mainly to the liver (n=7).

Discussion

In this study, a difference in the expression patterns of type I and IV collagen between non-liver metastatic CRC and liver-metastatic CRC was found. Type IV collagen was seen in close contact with the CRC cells at the invasive border of the tumour. It was earlier shown that type IV collagen induces proliferation of liver-metastasizing CRC cells, while the same was not observed for non-metastatic CRC cells (12). Furthermore, the importance of collagen IV in the process of liver metastases is supported by the in vivo findings of Burnier et al., where reducing the type IV collagen production in a liver metastatic murine carcinoma cell-line impaired the metastatic potential (13). Type IV collagen could be of importance for cancer cells escaping anoikis through an integrin-mediated binding of the cells to the ECM, thus providing the cells with important survival signals through the focal adhesion kinase (FAK)-mediated pathway (13). Additionally, Conti et al. showed that CLM express high levels of integrins, known to bind to both type I and IV collagen (12). The liver is particularly rich in type IV collagen in the liver sinusoids that are directly exposed to the portal blood from the colon. Thus, type IV collagen could be a matrix component that is well suited for the invasion and metastatic spread of CRC cells.

Furthermore, CRC cells that produce their own, or stimulate other cells to produce ECM components such as type IV collagen could have a growth-selective advantage. It has also been shown for type I collagen, that CRC cells display higher proliferation, chemoresistance, and survival (12). However, in our study, type I collagen was not seen in the vicinity of the cancer cells, but was rather more scattered throughout the DR in the primary tumour. Our findings in the primary CRC indicate that the risk of a subsequent CLM is related to the stromal composition of the primary tumour.

We were able to verify two of the liver metastatic growth patterns earlier described by Vermeulen et al. (11). The third, more unusual, replacement pattern was not identified in our material. A possible explanation for this could be the size of the cohort. There was no correlation between the stromal composition of the primary CRC and the growth pattern of the subsequent CLM. Thus the metastatic pattern in the liver could not be predicted by analysis of the primary tumour. However, the two groups differed significantly in the stromal composition of CLM. In desmoplastic CLM, mainly type IV collagen, but also type I collagen, was expressed intensely both inside the tumour as well as in the DR, separating the tumour from the normal liver parenchyma. In this DR, an intense inflammatory response was observed. In the pushing CLM, only a thin desmoplastic rim separated the cancer cells from the liver parenchyma, with a mild inflammatory infiltrate present in this area. This desmoplastic rim showed little or no expression of type I and IV collagen.

The pushing type of CLM was related to shorter overall survival, when compared to the desmoplastic CLM (Figure 4). However, at the same time the desmoplastic group had a significantly shorter time interval between CRC and CLM.

Table III. Metastatic sites in the pushing and desmoplastic groups.

<table>
<thead>
<tr>
<th></th>
<th>Pushing n=25*</th>
<th>Desmoplastic n=22*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deceased in CRC (n)</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td>Metastatic site</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colon (local recurrence)</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Liver</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Lungs</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Bone</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Other</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

*One patient was excluded due to a non-representative specimen of the CLM.

Figure 3. Expression patterns of collagens in normal liver tissue (A, D, G, J) and colorectal cancer liver metastases (CLM) with pushing (B, E, H, K) and desmoplastic growth patterns (C, F, I, L). H&E is haematoxylin-eosin stain; RET stains reticular fibers; COL-I visualizes type I collagen (brown); COL-IV visualizes type IV collagen (brown). Cancer cells are indicated by “T”, and the desmoplastic reaction by “DR”. In normal liver tissue, type I collagen is mainly found in the portal tracts (G), and type IV collagen is found in the liver sinusoids, as well as in the basal membranes of the vessels (J). The pushing growth pattern is characterized by the cancer cells pushing the liver parenchyma away, with little or no DR present (B, E). Only low levels of type I and IV collagen were found in this small DR (H, K). In the desmoplastic growth pattern, there was a rich DR separating the cancer cells from the liver parenchyma (C, F), with high collagen I and IV staining present (I, L). Histological analysis shows significantly higher collagen I (*p<0.001)(M) and IV (*p<0.001)(N) expression at the border of the desmoplastic type of CLM, compared to the border of the pushing type of CLM. Data for staining intensity and pattern are presented as the mean±SD (pushing CLM n=25 and desmoplastic CLM n=22, respectively).
which is a known factor for poor prognosis (14). One reasonable explanation for these findings is that liver surgery today cures up to 50% of patients with CLM (15) if the metastases are restricted to the liver. This is supported by our findings in the causes of mortality analysis for both types of CLM, as we found that patients with the pushing type of CLM displayed more disseminated cancer than those with the desmoplastic type of CLM (Table III). Interestingly, we found that nine patients with the pushing type of CLM developed lung metastases, but none did in the desmoplastic group. One explanation could be that desmoplastic CLM represents a more liver-specific metastatic disease, which can be surgically cured, while the pushing type of CLM could represent a type of cancer that does not need a DR to survive; thus, it is not as liver-specific, as demonstrated by the disseminated disease and higher mortality. Further support for this theory is that all patients in the desmoplastic group that died from their cancer had disease recurrence in the liver (n=7).

Another explanation for the difference in overall survival could be that the desmoplastic CLM with a dense DR is easier to detect with current diagnostic imaging compared to the pushing type that has no DR surrounding the metastases.

If this is the case, patients with desmoplastic CLM might be offered liver surgery at an earlier and potentially curable stage.

It has been proposed by Bissell et al. and experimentally shown that cancer cells without contact with a normal ECM become more malignant, and that the malignant potential of cancer cells can be reversed through more normalized ECM contact (10, 16). Additionally, there could be cancer cell-specific alterations that predict the metastatic pattern and the aggressiveness of the CLM. If this is the case, then the pushing type of CLM is a clearly more aggressive type of cancer and might need a different treatment strategy. Most interestingly, in patients who underwent re-resection of CLM due to recurrence, the CLM always displayed the same growth pattern as in the first metastasis, indicating that the growth pattern is constant and might be cancer cell type-specific or pre-determined by the host tissue.

The cohort of the study was selected based on the availability of matched tissue sections from the primary CRC and the subsequent CLM. This included patients diagnosed and treated over a 10-year period. During this time, chemotherapy treatments have changed and improved. Nevertheless, the distribution of desmoplastic and pushing...
types of CLM remained constant in the cohort over time. Additionally, the survival of patients with the pushing type of CLM remained poor despite these improvements.

In this study we have shown there to be a difference in stromal composition of mainly type IV collagen between non-metastatic CRC and liver-metastatic CRC. Our results indicate that the risk of being diagnosed with CLM is related to the stromal composition of the primary CRC. We verified two different types of CLM, with a desmoplastic growth pattern and a pushing growth pattern. These differ in their stromal composition and there was a remarkable difference in long-term survival and in the site of extrahepatic disease recurrence between the groups, indicating that the growth pattern of CLM is of significant clinical relevance.

Conflicts of Interest

The Authors declare no conflicts of interest.

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