Inflammation and uPAR-Expression in Colorectal Liver Metastases in Relation to Growth Pattern and Neo-adjuvant Therapy

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Abstract Proteolytic activity and inflammation in the tumour microenvironment affects cancer progression. In colorectal cancer (CRC) liver metastases it has been observed that three different immune profiles are present, as well as proteolytic activity, determined by the expression of urokinase-type plasminogen activator (uPAR). The main objectives of this study were to investigate uPAR expression and the density of macrophages (CD68) and T cells (CD3) as markers of inflammation in resected CRC liver metastases, where patients were neo-adjuvantly treated with chemotherapy with or without the angiogenesis inhibitor bevacizumab. Chemo-naïve patients served as a control group. The markers were correlated to growth patterns (GP) of liver metastases, i.e. desmoplastic, pushing and replacement GP. It was hypothesised that differences in proteolysis and inflammation could reflect tumour

specific growth and therapy related changes in the tumour microenvironment. In chemo-naïve patients, a significantly higher level of uPAR was observed in desmoplastic liver metastases in comparison to pushing GP ($p=0.01$) or replacement GP ($p=0.03$). A significantly higher density of CD68 was observed in liver metastases with replacement GP in comparison to those with pushing GP ($p=0.01$). In liver metastases from chemo treated patients, CD68 density was significantly higher in desmoplastic GP in comparison to pushing GP ($p=0.03$). In chemo and bevacizumab treated patients only a significant lower CD3 expression was observed in liver metastases with a mixed GP than in those with desmoplastic ($p=0.01$) or pushing GP ($p=0.05$). Expression of uPAR and the density of macrophages at the tumour margin of liver metastasis differ between GP in the untreated patients. A higher density of T cells was observed in the bevacizumab treated patients, when desmoplastic and pushing metastases were compared to liver metastases with a mix of the GP respectively, however no specific correlations between the immune markers of macrophages and T cells or GP of liver metastases could be demonstrated.

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Abbreviations

CRC	Colorectal cancer
EGFR	Epidermal growth factor receptor
5-FU	5-fluorouracil
GP	Growth pattern
IHC	Immunohistochemistry
mCRC	Metastatic colorectal cancer

MMP	Matrix metalloproteinase
PAI-1	Plasminogen activator inhibitor – 1
ROI	Region of interest
TRG	Tumour regression grade
uPA	Urokinase-type plasminogen activator
uPAR	Urokinase-type plasminogen activator receptor
VEGF	Vascular endothelial growth factor

Introduction

Metastatic disease is because of its high mortality rate a feared and serious consequence of cancer and a major challenge in medicine [1]. The interplay between tumour and stromal cells at the metastatic site is complex and several cell types are involved [2]. There has been a recent rise in interest in determining factors governing cancer immunity [3]. The tumour microenvironment consists of innate immune cells (macrophages, neutrophils, dendritic cells, mast cells and natural killer cells) [2, 4], adaptive immune cells (T and B lymphocytes) [5, 6] and stromal cells such as fibroblasts and endothelial cells [7, 8]. Accumulating evidence suggests that the tumour microenvironment plays a major role in the development of resistance to targeted therapies [9]. Thus, there is a great need to uncover biological features of the tumour microenvironment during antineoplastic treatment. This might also prove useful in the development of future response markers of targeted agents.

In colorectal cancer (CRC) the immune profile adds prognostic information [10]. Metastases from CRC are often confined to the liver, where additional stromal cells, such as host macrophages (Kupffer cells) and host myofibroblasts (hepatic stellate cells) are located [11, 12]. These cell types act together in a complex interplay including signals from cytokines and chemokines, shaping the microenvironment for metastatic tumour growth [12]. Additionally, several proteases capable of degrading the surrounding tissue such as matrix metalloproteinases (MMPs) and components of the plasminogen activation system (uPAR, uPA, PAI-1 and plasmin) are secreted by different cell types or released from tissue matrix upon degradation and tissue remodeling [13, 14]. uPAR has an ability to induce changes in the macrophages in the tumour microenvironment, which may be important for tumour progression [15]. Not only the cancer cells, but also the supporting stromal cells play a major role in tumour progression and further spread and the role of this extensive network of different cell types and signals must be revealed in order to enable development of future cancer therapies and response markers [16].

In CRC liver metastases, Vermeulen and colleagues described three different histological growth patterns (GPs): *desmoplastic*, *pushing* and *replacement* [17]. A mix of two

or three GPs is categorised as mixed GP. Patients resected for liver metastases with a desmoplastic GP have a superior survival in comparison to patients resected for liver metastases with a pushing, replacement or mixed growth pattern [18, 19]. The aim of this study was to investigate the correlation between GP and specific immune-relevant markers in the immediate tumour surroundings, specifically by evaluating the infiltration of macrophages and T cells and the urokinase-type plasminogen activation receptor (uPAR). uPAR which is of particular importance for tissue remodelling during cancer invasion, as the receptor is a prerequisite for pericellular plasmin formation [20]. In tissue uPAR is mainly expressed in macrophages and myofibroblasts and some cancer cells [21]. uPAR has been shown to correlate to a poor prognosis when analysed in blood and tumour/stromal tissue from CRC patients [22–25]. In the tissue uPAR is mainly expressed in stromal cells and some cancer cells in both CRC liver metastases [21, 26] and primary CRC [25].

The composition of immune cells in CRC liver metastases was studied by Halama and colleagues [27]. They observed three different immune profiles at the tumour margin of CRC liver metastases based on the number of T cells, B cells and macrophages. Another group, Katz et al., described the prognostic significance of T cell infiltration in CRC liver metastases [28, 29] observing that a high density of tumour infiltrating T cells correlated to a superior survival after liver resection. In contrast, the presence of regulatory T cells significantly hampered this positive effect on prognosis [30].

Macrophages have activity-dependent plasticity and capacity to adapt to the tumour microenvironment by regulation from cytokine and paracrine signalling [31], and thus play a role in the formation of metastases and in tumour progression [2]. In liver metastases the role of macrophages is yet not fully understood, but Kupffer cell-depletion has in an animal model demonstrated increased metastasis to the liver [32]. These studies suggest that the macrophages and T cells, as well as uPAR, in the tumour microenvironment are involved in the metastatic process. This prompted us to study the expression of uPAR as well as the density of macrophages and T cells in liver metastases from untreated and neo-adjuvantly treated patients, in an effort to identify some immune-related markers associated to GPs and therapy.

Patients and Methods

Patients

This retrospective explorative study included a consecutive series of 254 patients who were resected for colorectal liver metastases at Rigshospitalet, Copenhagen, Denmark, in the period 2007–2011. Patients resected for primary CRC, diagnosed with synchronous or metachronous liver metastases and

with no previous surgical treatment of CRC liver metastases and with no second primary cancer, were eligible for inclusion. Liver metastases with viable tumour cells and a representative tumour-liver interface were eligible for evaluation of uPAR, CD68, and CD3 in 237 of the 254 patients.

The patients were grouped according to neo-adjuvant treatment as follows: patients not having received neo-adjuvant treatment (untreated or chemo-naïve group, $n=149$), patients having received 5-FU based chemotherapy (chemotherapy group, $n=51$) and patients having received a combination treatment with 5-FU based chemotherapy and bevacizumab (chemotherapy plus bevacizumab group, $n=41$). Clinicopathological data were collected retrospectively by chart reviews.

The study was performed according to the Helsinki Declaration of the World Medical Association, The Committees of Health Research Ethics in the Capital Region of Denmark (H-2- 2011–045) and the Danish Data Protection Agency (2010-41-5623).

Histology

A total of 506 tissue blocks were available from 254 patients included, where 29 of the blocks had insufficient tissue for histological examination. The remaining 477 tissue blocks were available for histological examination. Sections of 3 μm were cut from the formalin fixed paraffin embedded tissue blocks. The sections were stained with Haematoxylin & Eosin (H&E) and Gordon-Sweet reticulin [26, 33]. A total of 45 tissue slides lacked a representative tumour-liver interface and were excluded from further analysis. The remaining 432 slides representing one or multiple liver metastases from 237 patients were included for additional immunohistochemical stainings. For patient with multiple CRC liver metastases, stainings against uPAR, CD68 and CD3 were performed on the liver metastasis with the least regression of tumour cells.

Antibodies

Rabbit polyclonal antibody (pAb) against uPAR (polyclonal antibody) has been described previously [34]. The following antibodies were purchased from Dako (Glostrup, Denmark): monoclonal antibodies against pan-cytokeratin (CK-pan, clone AE1/AE3) and CK20 (clone K_s20.8) were used for the detection of cancer cells and CD68 (clone PG-M1) for the detection of macrophages as well as a polyclonal antibody against CD3 (cat.no. A0452) for the detection of T cells. EnVision horseradish peroxidase Rabbit (K4003) secondary antibodies and an EnVision™ G|2 Double Staining Kit (K5361) were used and purchased from Dako.

Immunoperoxidase Staining (uPAR and CD3)

Three micrometer thick paraffin sections were cut from formalin fixed paraffin embedded tissue samples and used for immunoperoxidase staining. Antigen retrieval for the uPAR staining was performed by an enzymatic treatment with Proteinase K (5 $\mu\text{g}/\mu\text{l}$) in a proteinase K buffer (50 mM Tris-HCl, 50 mM EDTA, pH 8.0) at 37 °C for 15 min. Antigen retrieval for CD3 was performed by heat-induced epitope retrieval in a TEG buffer (10 mM Tris, 0.5 mM EGTA, pH 9.0) in 15 min using a T/T microwave processor at 98 °C (Milestone, Sorisul, Italy). Endogenous peroxidase activity was blocked by incubation with 1 % H_2O_2 for 15 min. The sections were washed in Tris-buffered saline (TBS, 50 mM Tris-HCl, 150 mM NaCl) containing 0.5 % Triton X-100 (TBS-T), and then mounted in Shandon racks with immunostaining cover plates (Thermo Shandon, Pittsburgh, PA) for further incubations. The antibodies were diluted in Antibody Diluent with Background-Reducing Components (S3022, Dako, Glostrup, Denmark) to a concentration of 2.88 $\mu\text{g}/\text{ml}$ (uPAR) or 0.55 $\mu\text{g}/\text{ml}$ (CD3). The antibodies were added to the slides and incubated overnight at 4 °C. The polyclonal antibodies were detected with EnVision™ Horseradish Peroxidase Rabbit. Each incubation step was followed by washes in TBS-T. The sections were developed with NovaRed (Vector laboratories, Burlingame, CA) and counterstained in Mayer's haematoxylin. Finally, slides were dehydrated and mounted using a Dako Coverslipper.

Double Immunostaining (CD68 and CK-pan/CK20)

Double stainings were done with the Envision™ G|2 Doublestain System Kit (K5361, Dako) following the manufacturer's instructions with some modifications. Antigen retrieval for the double staining of CD68 and CK-pan/CK20 was performed with Proteinase K (5 $\mu\text{g}/\mu\text{l}$) in a proteinase K buffer at 37 °C for 15 min. After pre-treatment, the sections were washed in TBS-T and manually mounted on Shandon racks with immunostaining cover plates (Thermo Shandon, Pittsburgh, PA). Endogenous peroxidase activity was blocked by Dual Endogenous Enzyme Block reagent provided in the kit for 15 min. The CD68 monoclonal antibody was diluted (0.30 $\mu\text{g}/\text{ml}$) in Antibody Diluent with Background-Reducing Components (S3022, Dako) and added to the sections. The first primary antibody was incubated for 2 h at room temperature. Afterwards the primary antibody was detected with Polymer/HRP (45 min) and developed with diaminobenzidine chromogene (DAB). Subsequently, Doublestain Block reagent was applied. The slides were incubated for 15 min. The second primary antibody, CK-pan/CK20 was diluted (CK-pan; 0.36 $\mu\text{g}/\text{ml}$, CK20; 0.23 $\mu\text{g}/\text{ml}$) in Antibody Diluent with Background-Reducing Components and added to the slides. The second primary antibody was incubated

overnight at 4 °C. Subsequently the Rabbit/Mouse (LINK) reagent was applied to the slides and incubated for 30 min. The second primary antibody was detected with Polymer/AP reagent for 45 min. Visualisation was performed with Permanent Red. Each incubation step was followed by washes in TBS-T and the slides were counterstained with Mayer's haematoxylin. Finally, slides were dehydrated in an oven at 60 °C for 1 h before coverslips were mounted with pertex using a CoverSlipper from Dako.

Histopathologic Assessment

Liver Metastasis Growth Patterns and Tumour Regression Grade

H&E and reticulin stained slides were used for the assessment of GP, as described previously [26]. The H&E stained slides were also used for assessment of tumour regression grade (TRG) by using the Rubbia Brandt classification [35]. The Rubbia-Brandt tumour regression grade classification is divided into five scores, depending on the amount of residual cancer cells, necrosis and fibrosis. The TRG score 5 is given to metastases where no signs of regression (i.e. necrosis and fibrosis) appear. TRG score 4 is given to liver metastases with some necrosis and fibrosis, but liver metastasis mainly consists of tumour cells. TRG score 3 is given for liver metastases where an almost equal amount of tumour cells and necrosis/fibrosis appear. TRG score 2 is given when few residual cancer cells are left in a necrotic and fibrotic metastasis. TRG score 1 is given when no residual tumour cells are observed, only necrosis and fibrosis. For patients with multiple CRC liver metastases, the metastasis with the highest TRG score was used for further analysis. The metastases were identified in a previous study [36].

uPAR-Expression

The scoring of uPAR in liver metastases has previously been described [26]. The level of uPAR-expression was divided into five scores, based on the percentage of uPAR positive cells at the tumour margin: score 0: no uPAR positive cells, score 1: less than 5 % uPAR positive cells, score 2: between 5 and 10 % uPAR positive cells, score 3: between 10 and 30 % uPAR positive cells, score 4: above 30 % uPAR positive cells. For desmoplastic metastases the scoring of uPAR was performed between the tumour cells and the rim of collagen (Fig. 1). For pushing, replacement and mixed GP uPAR expression was evaluated at the tumour periphery. uPAR positivity was also found in neutrophils throughout the tissue, which served as a positive internal control [37].

Macrophages and T Cells

Digital image analysis was performed by using algorithms from the Visiomorph™ software (Visiopharm, Hørsholm, Denmark). The algorithm for macrophages and T cells respectively, was developed. For macrophages, the double staining of CD68 and CK-pan/CK20 ensured that the tumour margin could easily be identified (Fig. 2). For T cells, the tumour margin could also easily be identified as the CK-pan/CK20 stained slides were neighbouring sections to the CD3 stained slides. The scoring was performed at the tumour margin of the metastasis. A line was drawn at the tumour margin and a region of interest (ROI) formed by a digital classifier with the broadness of 500 µm, including 250 µm of the tumour periphery and 250 µm of the stromal tissue or the liver parenchyma closest to the tumour periphery. When the ROI was calculated and drawn by the computer, an algorithm for recognition of DAB positive CD68 and NovaRed positive CD3 was running. The outcome was a measurement of CD68 or CD3 positive cells within the area of the ROI. The measurement was expressed as the number of positive CD68 or CD3 positive pixels in relation to the ROI (pixels per µm²). This fraction was used for further calculations. The calculation of CD68 and CD3 was done in separate analyses. For pushing, replacement and mixed GPs the line was drawn at the tumour margin, while in the desmoplastic metastases a line between the collagen rim and the liver parenchyma was drawn, since the inflammatory infiltrate was denser at the collagen/liver interface. The uPAR-expression and the inflammatory infiltrate were therefore not scored at the same site in the desmoplastic metastases.

Statistics

For statistical analyses the log-linear general model was used for the dependent variables. A *p*-value <0.05 was considered statistically significant. The Spearman correlation analysis was used for comparisons. The SPSS version 19.0 as well as the S.A.S. software version 9.3 (S.A.S. Institute, Cary, N.C., USA) was used for statistical analyses.

Results

Tumour regression grades (TRG) of the liver metastases examined were determined in a previous study [36]. In patients with multiple liver metastases the metastasis with the highest TRG score was analysed and GP characterised. In total liver metastases from 237 patients were characterised: desmoplastic GP 70 patients (29.5 %), pushing GP 88 patients (37.1 %), replacement GP 34 patients (14.3 %) and mixed GP in 45 patients (19.0 %). Examples of the GPs of the CRC liver

Fig. 1 Demonstration of CRC liver metastases GPs: desmoplastic (panel **a**), pushing (panel **e**) and replacement (panel **i**) in relation to uPAR expression (panel **b**, **f** and **j**), macrophage density (panel **c**, **g** and **k**) as well as T cell density (panel **d**, **h** and **l**). Bar ~200 μ m

metastases, desmoplastic, pushing and replacement are seen in Fig. 1a, e, and i as H&E stained sections.

uPAR-Expression

uPAR immunoreactivity was primarily located in macrophages and myofibroblasts as well as a few cancer cells at the tumour margin (Fig. 1b, f, and j). uPAR immunoreactivity was also found in metastases with central necrosis. In liver metastases from chemo-naïve patients a significantly higher expression of uPAR was observed in desmoplastic GP in comparison to pushing ($p=0.01$) or replacement GP ($p=0.03$) (Fig. 3a). No significant difference of uPAR-expression according to GP was observed in liver metastases from patients in the chemotherapy group or the bevacizumab group (Fig. 3a).

Macrophages

Macrophages were located at the tumour margin, but also between tumour glands (Fig. 1c, g, and k). Kupffer cells were also positive for CD68. The digital scoring of CD68 positivity

(Fig. 2) between two observers had a systematic difference of 10 %, but the inter-observer correlation was high ($r^2=0.90$).

Liver metastases with replacement GP from patients in the untreated group had significantly higher CD68 level in comparison to those with pushing GP ($p=0.01$) (Fig. 3b). Macrophage density did not differ significantly between any of the other GPs. Furthermore, no differences were observed between GPs in the liver metastases from neo-adjuvantly treated patients. It was found that macrophage density was significantly higher in the chemo-naïve versus chemo treated group ($p=0.02$). No difference of CD68 density was observed between chemo-naïve versus chemo plus bevacizumab treated or between chemo versus chemo plus bevacizumab treated. A correlation was observed between uPAR and CD68, $r^2=0.29$, $p<0.01$.

T Cells

T cells were located at the tumour margin as part of an immune infiltrate. The CD3 positivity (Fig. 1d, h, and l) was significantly higher when metastases had a desmoplastic GP in comparison to a mixed type ($p=0.01$) as well as a pushing GP in comparison to a mixed type ($p=0.05$) in chemotherapy plus

Fig. 2 Digital CD68 algorithm by Visiopharm software. In panel **a** the region of interest of 500 μ m was determined at the tumour margin. In panel **b**, **c** and **d** the algorithm is shown at a higher magnification (20X objective). Macrophages are marked as yellow pixels. Bar ~50 μ m

Fig. 3 The level of uPAR (panel **a**), macrophages (panel **b**) and T cells (panel **c**) is represented for liver metastases with different growth patterns in relation to therapy: untreated (black boxes), neo-adjuvantly treated with chemotherapy (red boxes) and neo-adjuvantly treated with chemotherapy

and bevacizumab (green boxes). The box plots represent the interquartile range, while lines are 1.5X the interquartile range. Dots represent values outside the interquartile plus interquartile times 1.5X range

bevacizumab treated patients (Fig. 3c). The density of CD3 positive cells did not differ significantly between mixed and replacement GP in the chemotherapy plus bevacizumab group. No differences of CD3 according to GP were observed in liver metastases from the chemo-naïve patients. As in liver metastases from chemo-naïve patients, the density of CD3 positive cells at the tumour margin of liver metastases from chemotherapy treated patients did not differ between GPs. No significant difference of T cell density was observed between therapy groups. No significant correlations were found between uPAR and CD3 positive cells ($p=0.57$) or between CD3 and CD68 positive cells ($p=0.40$).

Discussion

In chemo-naïve patients we observed a higher uPAR-expression in the tumour periphery of desmoplastic liver metastases compared to liver metastases with pushing or replacement GP. This confirms our earlier findings [21, 26]. A similar difference was, however, not found in metastases from patients treated neo-adjuvantly. uPAR is up-regulated at the invasive front of the tumour in primary CRC and a high uPAR expression has been correlated to an inferior outcome [25]. Therefore it is interesting that most uPAR is found in liver metastases with desmoplastic GP as this type of metastases has been shown to be associated to a longer recurrence free survival independent of neo-adjuvant therapy [33]. It is very likely that uPAR in desmoplastic liver metastases is involved in a tissue remodelling process. Furthermore, the exact role of the desmoplastic stroma in CRC liver metastases is not known. The desmoplasia could be a host specific reaction acting as a barrier for further cancer cell infiltration of the liver parenchyma. The higher uPAR expression in desmoplastic metastases may be a secondary reaction of the tumour cells

to the desmoplasia. The reason why the uPAR expression did not differ between GPs in patients given neo-adjuvant treatment is unknown, but therapy may induce remodelling processes in tumour tissue in general.

Interestingly, we observed a significantly higher level of macrophages in liver metastases from chemo-naïve patients in comparison to patients given neo-adjuvant chemotherapy before liver resection, but not in comparison to liver metastases from patients who received chemotherapy plus bevacizumab. That the level of macrophages is higher in the chemo-naïve patients may reflect the fact that bone marrow derived cells are recruited to the tumour microenvironment, in order to constitute a tumorigenic environment [38]. This recruitment may be disturbed by cytotoxic chemotherapy [39]. Targeted therapies with monoclonal antibodies may restrict tumour growth by macrophage cytotoxic properties [40] as well as by tumour-specific cytotoxic T cell responses [41]. Monoclonal antibodies may modify human immune responses [42, 43]. This may explain the finding of a denser macrophage infiltrate of liver metastases in the chemotherapy plus bevacizumab treated patients in comparison to the chemo treated patients, however, the difference was not significant. In animal models it may be possible to investigate the macrophage density before and after monoclonal antibody therapy, which could tell us whether immune responses arise as a reaction to tumour infiltration or whether the responses arise because of the therapy with monoclonal antibodies. To authors knowledge, no optimal animal model exists for CRC liver metastases, where all three GPs can be studied in the same model. Because we did not have “before” and “after” treatment observations, we used the chemo-naïve patients as a reference group for comparison of CD68 levels. The macrophage density of the GPs did not differ between therapy groups.

Three different immune profiles at the tumour margin of CRC liver metastases have been described by Halama et al. [27]. Our research hypothesised that these profiles corresponded to the three different GPs. This, however, could not be demonstrated. One reason could be that patients in the Halama study received neo-adjuvant chemotherapy with either bevacizumab or cetuximab, whereas the present study included both treated and untreated patients. It is highly likely that different targeted therapies is of importance for the resulting inflammatory response, as both bevacizumab and cetuximab are monoclonal antibodies against the vascular endothelial growth factor and the epidermal growth factor receptor, respectively. One could speculate whether the immune cells induce a specific GP or not, but our findings do not support such a theory. In future studies it would be valuable to study metastases from chemonaive patients as this is most likely close to tumour growth behaviour, while metastases from treated patients may include different resistance mechanisms, making tumour growth studies as well as immune response studies more difficult to interpret.

The uPAR-expression differed between desmoplastic and the non-desmoplastic GP, pushing and replacement, in untreated patients, but not in neo-adjuvantly treated patients. Macrophages (CD68) were elevated in liver metastases of replacement GP in comparison to non-replacement GPs in the untreated, but not in the neo-adjuvantly treated. T cell density did not differ significantly between GPs or between therapy groups. GPs are intriguing, but do not seem to be correlated to specific levels of macrophages and T cells in the periphery of CRC liver metastases.

Inflammatory characteristics and remodelling in response to targeted therapy are important, but also very difficult to investigate as the immune responses are complex. That macrophage density is lower in the liver metastases from patients who received neo-adjuvant cytotoxic chemotherapy in comparison to liver metastases from chemonaive as well as from patients who received neo-adjuvant chemotherapy and bevacizumab is an interesting finding and needs further investigations. Furthermore, the immune response after targeted therapies with monoclonal antibodies would be relevant to study in further details.

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