

Localization and Density of Immune Cells in the Invasive Margin of Human Colorectal Cancer Liver Metastases Are Prognostic for Response to Chemotherapy

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Abstract

Analysis of tumor-infiltrating lymphocytes (TIL) in primary human colorectal cancer (CRC) by *in situ* immunohistochemical staining supports the hypothesis that the adaptive immune response influences the course of human CRC. Specifically, high densities of TILs in the primary tumor are associated with good prognosis independent of other prognostic markers. However, the prognostic role of TILs in metastatic CRC lesions is unknown, as is their role in response or resistance to conventional chemotherapy. We analyzed the association of TIL densities at the invasive margin of CRC liver metastases with response to chemotherapy and progression-free survival in a set of 101 large section samples. High-resolution automated microscopy on complete tissue sections was used to objectively generate cell densities for CD3, CD8, granzyme B, or FOXP3 positive immune cells. A predictive scoring system using TIL densities was developed in a training set and tested successfully in an independent validation set. TIL densities at the invasive margin of liver metastases allowed the prediction of response to chemotherapy with a sensitivity of 79% and specificity of 100%. The association of high density values with longer progression-free survival under chemotherapy was statistically significant. Overall, these findings extend the impact of the local immune response on the clinical course from the primary tumor also to metastatic lesions. Because detailed quantification of TILs in metastatic lesions revealed a strong association with chemotherapy efficacy and prognosis, we suggest that the developed scoring system may be used as a predictive tool for response to chemotherapy in metastatic CRC. *Cancer Res*; 71(17); 1-8. ©2011 AACR.

Introduction

Metastatic colorectal cancer (CRC) accounts for more than 55,000 deaths per year in the United States alone, with the liver

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being the most frequent site of distant metastases (1). In 20% of patients, liver metastases can be resected with curative intent (2). Neoadjuvant treatment can improve resectability, but the majority of the patients have nonresectable liver metastases and receive palliative chemotherapy (3). The overall prognosis of patients receiving systemic palliative chemotherapy is very limited with a median overall survival of approximately 24 months (4). Modern chemotherapy regimens include monoclonal VEGF or epidermal growth factor receptor (EGFR) antibodies and lead to objective response rates of around 40% to 60% (4), whereas a large proportion of patients experience treatment-related side effects without any clinical benefit. The only established predictive markers are *K-Ras* mutations in the tumor, which predicts nonefficacy to treatment with EGFR antibodies (5). So far, no biomarkers are available that help to select patients that are likely to respond to chemotherapy. Immune cells infiltrating or surrounding colorectal primary tumors (6) and especially tumor-infiltrating lymphocytes (TIL) represent important prognostic factors (7, 8). High densities of TILs were shown to be correlated with an improved survival in patients with primary CRC (9–14). In metastatic lesions, the impact of immune infiltrates on the

clinical course of CRC has not been analyzed systematically so far. The role of specific immune cell subpopulations and the role of the immune system in mediating response or resistance to conventional chemotherapy in metastatic CRC is also not clear (15). Only in few patients, a diagnostic excision or biopsy is taken from the liver metastasis before chemotherapy is initiated, so availability of material is limited. Examining a small series of samples from primary colorectal tumors and corresponding liver metastases, we have found marked differences between infiltrate densities on the invasive margin of liver metastases (16). Following these preliminary observations, we have now analyzed TIL infiltrates in a large cohort of metastatic CRC patients using objective high-throughput quantification of immune cells on large whole slide sections (17–19). We describe here for the first time a strong association between immune cell density in the invasive margin of CRC liver metastases and chemotherapy response, highlighting the importance of the local immune response in metastatic CRC.

Materials and Methods

Study design and concept

Records of patients with metastatic CRC who underwent diagnostic excision (or primary resection) of liver metastases or liver biopsy at different high-volume centers between 1998 and 2008 were reviewed and suitable samples were retrieved. Immune cell infiltrates at the invasive margin of CRC liver metastases were analyzed and correlated with response to chemotherapy in a total of 101 patients with metastatic CRC. For 33 patients, material from diagnostic excisions (during resection of the primary tumor or laparoscopy) and detailed follow-up, including progression-free survival under chemotherapy, were available. The samples from this patient cohort were used as a training set (see Supplementary Information) to generate a scoring system ("density score") to be used to predict treatment response. Samples from 68 other patients were used as a validation set. For the latter, only dichotomous response data were available. Patient data and samples from metastatic CRC patients without treatment were not available. Immune cell antigens were selected on the basis of literature data, where an association with prognosis but not with treatment response was already identified and independently confirmed in primary CRC (CD3, CD8, and granzyme B). The density and distribution of FOXP3-positive regulatory T cells in metastatic CRC is still unclear and was therefore also analyzed. The term "prognostic marker" is used throughout this article according to the REMARK Guidelines.

Clinical data

The study was approved by the local ethics committees. One hundred one tumor samples of metastatic CRC liver metastases were used for this study after obtaining signed informed consent from all patients. Progression-free survival was defined as the time from the beginning of palliative chemotherapy until disease progression (failure) and was only available for the training set of 33 patients, consisting of samples from the University Medical Center Heidelberg and

the SLK Klinikum Heilbronn. No patient died during the observed period. Follow-up for each patient included the complete time period until remission or disease progression occurred and overall survival.

The independent validation set consisted of samples from patients treated at the aforementioned facilities (22 patients) and from the prospective multicenter CELIM trial (46 patients), investigating neoadjuvant treatment of nonresectable liver metastases. Patients in the CELIM trial had agreed to a needle liver biopsy or the use of previously acquired biopsies or sections from diagnostic excisions from the liver metastases for EGFR immunohistochemistry and for further scientific evaluation. Details of this trial are reported elsewhere (3). All 101 patients received chemotherapy (irinotecan-based, platinum-based, 5-FU) either with or without concomitant antibody therapy (cetuximab or bevacizumab; see Supplementary Information).

In this study, patients were included with stage UICC IV CRC after metastatic disease was histologically proven. Included samples either consisted of large liver biopsies and partially resected liver metastases ("technically nonresectable," see Supplementary Fig. S1) or diagnostic excisions. All tissue investigated had to contain at least more than 1 mm² of evaluable invasive margin (see Supplementary Fig. S2). All patients received (palliative) chemotherapy that was begun within 8 weeks after diagnosis. So the patients in this study are from a rare, but clinically highly informative, subgroup among all patients with CRC. Other patient inclusion criteria were a Karnofsky performance score of at least 80% and adequate hepatic, renal, and bone marrow function. Patients with synchronous liver metastasis were included if the primary tumor had been resected before chemotherapy. Supplementary Table S1 shows the clinical characteristics of both sets of patients. Before start of chemotherapy and every 4 cycles (usually 2 months), staging was conducted by computed tomography or MRI. Response to chemotherapy was evaluated according to Response Evaluation Criteria in Solid Tumors (RECIST). In our investigation, response was defined as partial (or complete) remission or progressive disease. Patients with stable disease after initiation of chemotherapy were further monitored under the same treatment until either disease progression or remission occurred. Evaluations were conducted by review of blinded radiologists or as reported previously (3) and best (unconfirmed) responses were used in this analysis. Tumor samples were typed for microsatellite instability using the polymorphic markers BAT25, BAT26, and CAT25 as described earlier (20) and all metastases were of microsatellite stable status.

Immunohistochemical staining

Tissue specimens were immunohistochemically analyzed for their infiltration with CD3, CD8, granzyme B, and FOXP3-positive T cells. Tissue sections (4 μm) were prepared from formalin-fixed, paraffin-embedded tissue. After deparaffinization and rehydration, the slides were boiled in 10 mmol/L citrate buffer (pH = 6) for 15 minutes to retrieve the antigens. The endogenous peroxidase activity was blocked by incubation with 0.6% H₂O₂ in methanol for 20 minutes. The sections were

blocked with 10% normal horse serum (VECTASTAIN Elite ABC kit; Vector Laboratories). Mouse monoclonal antibodies recognizing human CD3 ϵ (1:50 dilution; clone PS1; Acris), CD8 (1:40 dilution; clone 4B11; Novocastra), GrB (1:50 dilution; clone 11F1; Novocastra), and FOXP3 (1:100 dilution; clone 236A/E7; Abcam) were applied as primary antibodies at room temperature for 2 hours. The slides were incubated with a biotinylated secondary antibody (1:50 dilution; horse-anti-mouse IgG; VECTASTAIN Elite ABC kit; Vector Laboratories) for 30 minutes at room temperature and AB reagent was applied according to the manufacturer's instructions (VECTASTAIN Elite ABC kit; Vector Laboratories). The antigen detection was conducted by a color reaction with 3,3'-diaminobenzidine (DAB + chromogen; Dako Cytomation). The sections were counterstained with hematoxylin (AppliChem) and mounted with Aquatex (Merck).

Evaluation of immunohistochemical variables

The number of stained immune cells was counted using a computerized image analysis system consisting of an NDP Nanozoomer from Hamamatsu Photonics attached to a personal computer. Complete microscopic images of full tissue sections were automatically obtained for later automatic or visual analysis (virtual microscopy), allowing large scale histologic evaluation with high precision across the complete section. Thus, varying cell densities across the complete tissue section can be measured objectively. In this analysis, the average cell density across the measured region was used for analysis. The invasive margin was defined as a region of 500 μm width on each side of the border between malignant cells ("metastasis" and peritumoral stroma) and liver tissue (see Supplementary Information).

Manual evaluation of stained immune cells was conducted (in duplicates) without knowledge of the clinicopathologic data by 2 independent observers. Variations in the enumeration within a range of 5% were reevaluated and a consensus decision was made. The results were expressed as the mean of positive stained cells per mm^2 . A total of 1,002 mm^2 of tissue surface area was analyzed, with a minimum of 1 mm^2 of invasive margin per sample. Manual cell counts were reassessed with a specifically developed software program (VIS software suite; Visio-pharm) to measure cell densities across a given region of interest (on average 10 mm^2 , with up to 40 mm^2) as reported previously (17). The automated cell counts confirmed the manual cell counts, so that the automated quantification was applied to the validation set of 68 patients, avoiding observer bias (17). All evaluations were visually checked for consistency.

Statistical analyses

Primary aim of the study was to evaluate the use of TIL densities to predict response to chemotherapy. A training set of 33 patients was considered to derive a prediction model based on CD3, CD8, and granzyme B cell densities as input variables of the model. In the literature, the prognostic role for FOXP3 cell densities is unclear for

metastatic CRC, therefore these cell densities were analyzed in a second exploratory step (see Supplementary Information). For the other immune cell surface markers, we applied recursive partitioning by conditional inference trees to develop a scoring system to be used as prediction rule (21). The algorithm works as follows: (i) Test the global null hypothesis of independence between any of the input variables and the response. Stop if this hypothesis cannot be rejected. Otherwise select the input variable with strongest association to the response. This association is measured by a P value corresponding to a test for the partial null hypothesis of a single input variable and the response. (ii) Implement a binary split in the selected input variable. (iii) Recursively repeat steps (i) and (ii). An independent set of 68 patients was used to evaluate this prediction model. A secondary analysis was conducted to analyze the prognostic value of the derived scoring system with respect to time to progression by proportional hazards regression. Survival curves were computed using the Kaplan–Meier estimator.

To compare clinicopathologic characteristics between the training and the validation set, the exact Mann–Whitney test was used for continuous variables. To compare categorical data, Fisher's exact test was applied.

All statistical analyses were conducted with R version 2.11.0 together with the R package party, version 0.9 (22).

Results

The evaluation of the invasive margin of metastatic liver lesions showed striking variations in TIL densities at the invasive margin between different patients (Fig. 1) as was noted previously (16). While the center of the metastasis often contains large areas of necrosis, making objective evaluations difficult, the invasive margin with its clear border showed a clear heterogeneity of TIL densities between patients also in this larger patient cohort. For the invasive margin, in the training set of 33 patients, a scoring system ("density score") was developed to differentiate between patients with different levels of TIL densities and to test whether TIL densities were related to chemotherapy response. An independent set of 68 patients ("validation set") was used to validate the scoring system with respect to response prediction. Classification by recursive partitioning of the observed TIL densities (CD3, CD8, and granzyme B) of the 33 metastatic lesions classified as responder or nonresponder in the training set revealed the following prediction rule (see Supplementary Fig. S3): Patients having a CD3 cell count above 572 cells/ mm^2 and either a CD8 density of higher than 170 cells/ mm^2 or a granzyme B density of higher than 23 cells/ mm^2 are predicted to respond to therapy. For practical reasons, the finally derived rule required a CD3 cell count above 600 cells/ mm^2 and either a CD8 density of higher than 200 cells/ mm^2 or a granzyme B density of higher than 25 cells/ mm^2 . To incorporate this prediction rule into a scoring system, a density score was generated. A CD3 density above 600 cells/ mm^2 receives double impact, CD8 and GranzB have single weight ("2+1+1"). The range of the score is therefore from 0 to 4, where patients with a score from 0 to 2

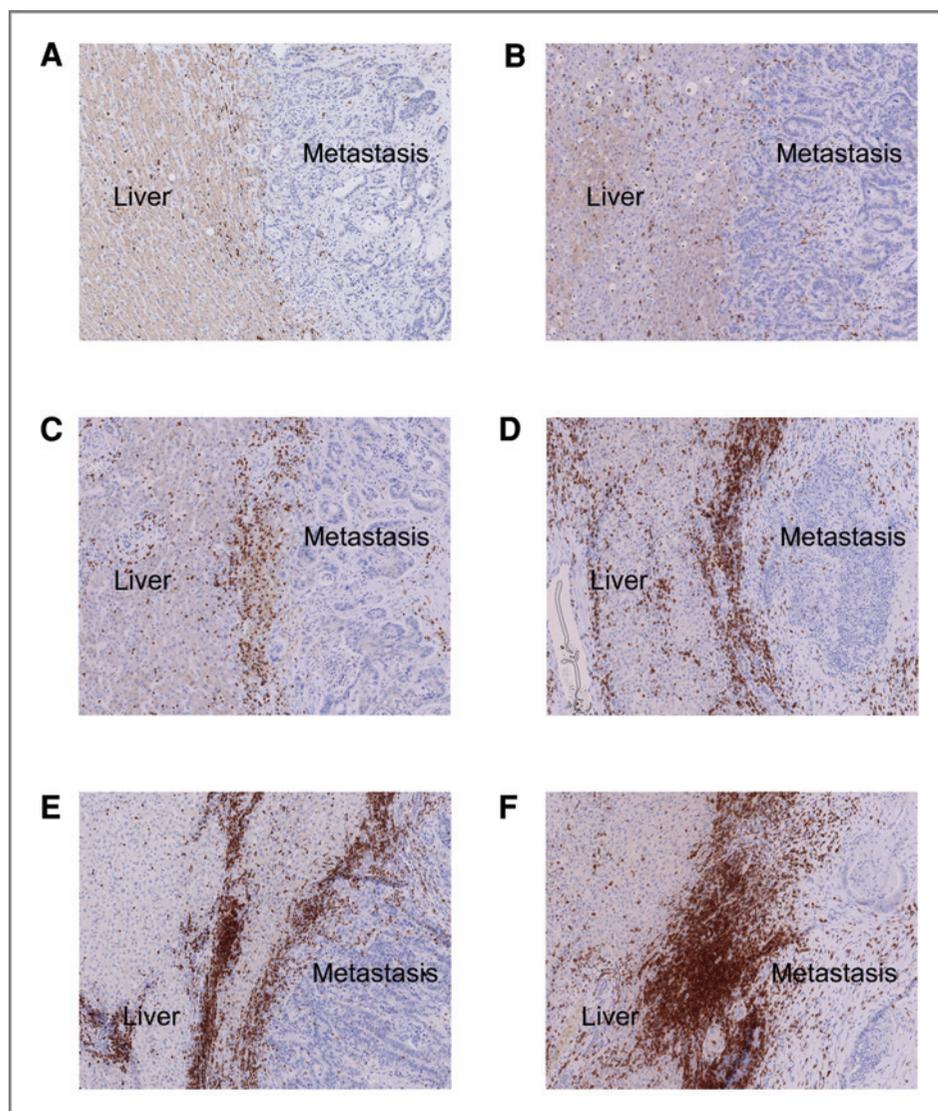


Figure 1. Immunohistologic images of liver metastases (digital magnification, 10 \times), CD3 staining (dark red), and hematoxylin counterstaining. A and B, the invasive margins from 2 different metastases from 2 different patients with a density score of 0 to 2 ("low infiltrate density," progression-free survival 2 and 4 months). C and D, 2 metastases with a density score of 3 ("high density," progression-free survival 10 and 11 months). E and F, metastases from 2 different patients with a density score of 4 ("high density," progression-free survival 18 and 24 months).

("low density") are predicted to be nonresponders. The addition of granzyme B and CD8 evaluation in the scoring system allowed a more reliable classification than cell counts for CD3 alone.

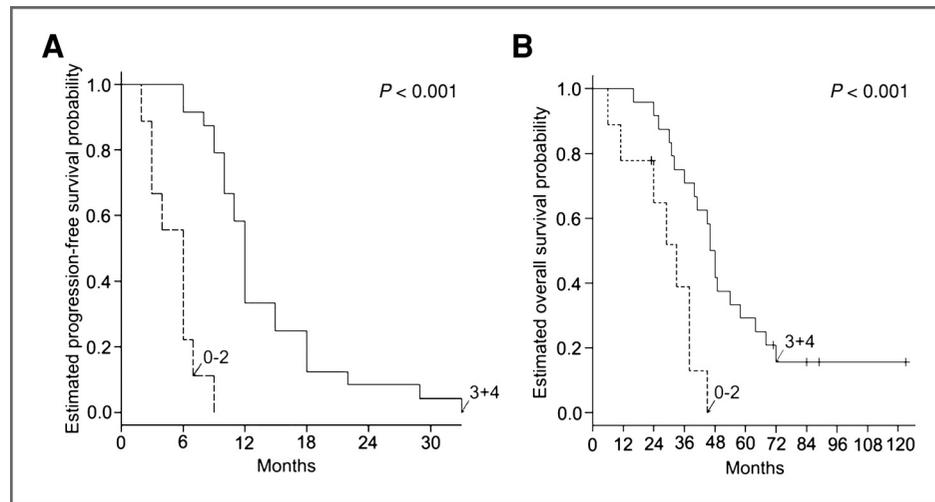
The distribution of clinical parameters was investigated: age and gender as well as chemotherapeutic regimen distributions were similar between the training and validation set. Information with regard to the distribution of age, gender, localization, immune cell distribution, microsatellite status, antibody treatment, and chemotherapeutic regimens can be found in Supplementary Table S1.

To further corroborate these findings, the use of the scoring system to predict progression-free survival in the training set was analyzed. Despite initial success of chemotherapy, patients can have short intervals to disease progression and response to chemotherapy not necessarily leads to prolonged treatment periods with the same treatment regimen (23). With respect to progression-free survival, significant differences

between the groups with density scores from 0 to 2 ("low density") and 3 + 4 ("high density") were found (training set: $P < 0.001$). Figure 2A shows the corresponding Kaplan–Meier plot of groups with scores 0 to 2 and 3 + 4. Estimated HR for patients with score 3 or 4 was 0.06 (95% CI: 0.02–0.20) in the training set. Similar results could be found in the overall survival analysis (Fig. 2B), where a statistically significant difference was found between the 2 groups (log-rank test, $P < 0.001$). Including additional covariates (age, gender, chemotherapy, and antibody treatment) did not change the results for the scoring system. The scores of all 33 patients and the corresponding density score groups are shown in Figure 3.

On the basis of the above developed scoring system, the response of patients from the validation set could be predicted with high accuracy in the validation set. The estimated prediction error was 14.7% with a 95% CI ranging from 8.2% to 25.0%. All 21 nonresponders of the validation set were

Figure 2. Kaplan–Meier plots of estimated (A) progression-free survival probabilities and (B) overall survival probabilities across the groups with scores 0 to 2 ("low density") and with scores 3 + 4 ("high density").



correctly predicted, specificity being 100% (95% CI: 85%–100%). No nonresponder was in the group of patients predicted to have a response to chemotherapy. Ten of the 47 patients who responded to treatment were predicted to be nonresponders, sensitivity therefore being 79% (95% CI: 65%–88%). This might be explained by the high rate of needle biopsies among these misclassified patient samples (see Supplementary Information).

Additional analysis of FOXP3-positive regulatory T-cell densities in a subset of 20 patients from the training set revealed that their number is generally low in the invasive margin of metastatic CRC liver metastases (see Supplementary Information). CD3-positive T cells outnumbered FOXP3-positive lymphocytes by 50–100 to 1. FOXP3-positive regulatory T cells also did not show differences in distribution between responders and nonresponders.

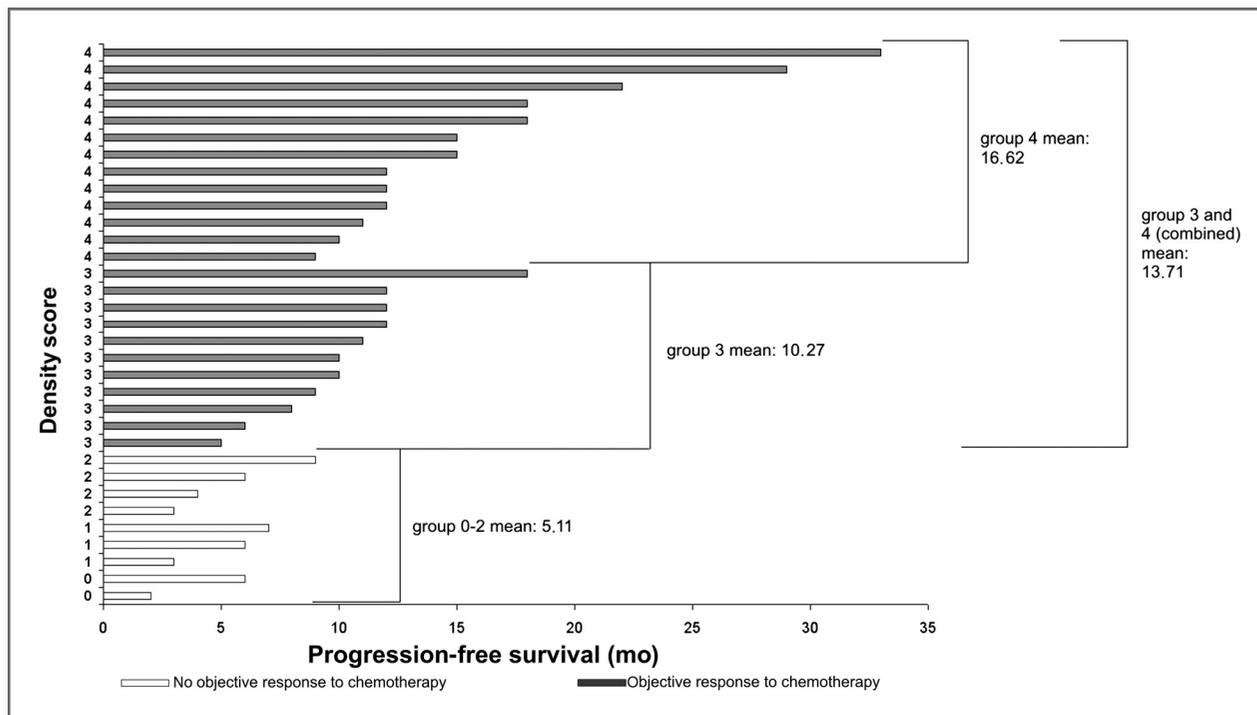


Figure 3. Progression-free survival of patients according to the density scoring system (based on the analysis of the invasive margin of the liver metastases). Each horizontal bar represents a single patient; the length of the bar represents the individual progression-free survival (in months). The group with score 0 to 2 ("low infiltrate density") corresponds to the patients with no response to chemotherapy (indicated by open bars). Average progression-free survival in months for each group is displayed (brackets show the respective group); differences between the groups were statistically significant (see Results).

Discussion

The densities and localization of TILs in metastatic liver lesions of CRC have not been investigated in detail. While findings reported by Galon et al. (9) corroborated the prognostic significance of TILs in the primary CRC tumor across all tumor stages and showed a clear role for effector T cells, the relation of infiltrate densities and response to chemotherapy in patients with metastatic CRC (stage IV) was not analyzed. Likewise, the prognostic impact of infiltrates at the site of the metastases has not been investigated in detail so far. As patients with metastatic CRC frequently undergo surgery to remove the primary tumor, the metastases are often the remaining tumor tissue.

Here, we show for the first time a strong association between the local immune cell profile and chemotherapy outcome in CRC liver metastases. This clearly extends the observed role for the immune system in primary CRC also into metastatic lesions and further into chemotherapy efficacy. For the first time in CRC, ample tumor tissue was analyzed with the remainder of the tumor tissue being monitored during therapy. Previous work is mainly based on small tissue samples, but clear associations of heterogeneous immune cell densities and clinical aspects require a large-scale tissue evaluation (see Fig. 4). This situation is also in contrast to other work that analyzed only the adjuvant situation, where the complete metastatic burden is removed and any adjuvant treatment is given without knowledge of remaining tumor cells (24). Another advantage of our approach was that the here reported association was validated in an independent patient cohort. Also, the immune cell density showed a significant prognostic effect on the progression-free survival under chemotherapy. T-cell densities at the invasive margin of metastatic lesions showed considerable differences between patients who responded to chemotherapy versus nonresponders (16). The invasive margin of the metastatic liver lesion and the invasive margin of the primary tumor both constitute the boundary where malignant cells are in contact with presumably nonmalignant tissue. While metastatic malignant cells certainly have a different phenotype from nonmetastatic tumor cells, an immunologic distinction (e.g., in immune cell activation) has not been shown between metastatic and nonmetastatic cells. Our data show that response to chemotherapy is associated with a high T-cell density at the invasive margin of metastatic lesions. In addition, there was a significant association of high T-cell numbers with good prognosis (Figs. 2 and 3). The relative low percentage of nonresponders to chemotherapy (27%) in our cohorts may be surprising but could be explained by a selection bias for patients undergoing surgery for presumably resectable metastatic disease. Furthermore, the validation cohort consisted mainly of patients from a large multicenter trial (3) in which a high response rate was reported that may explain the above observation. The presented patient cohort is unique, as larger numbers of samples from diagnostic excisions of liver metastases with ample invasive margin are difficult to obtain. Standard clinical procedure includes only fine-needle biopsies of (suspected) metastases, if any at all. These standard

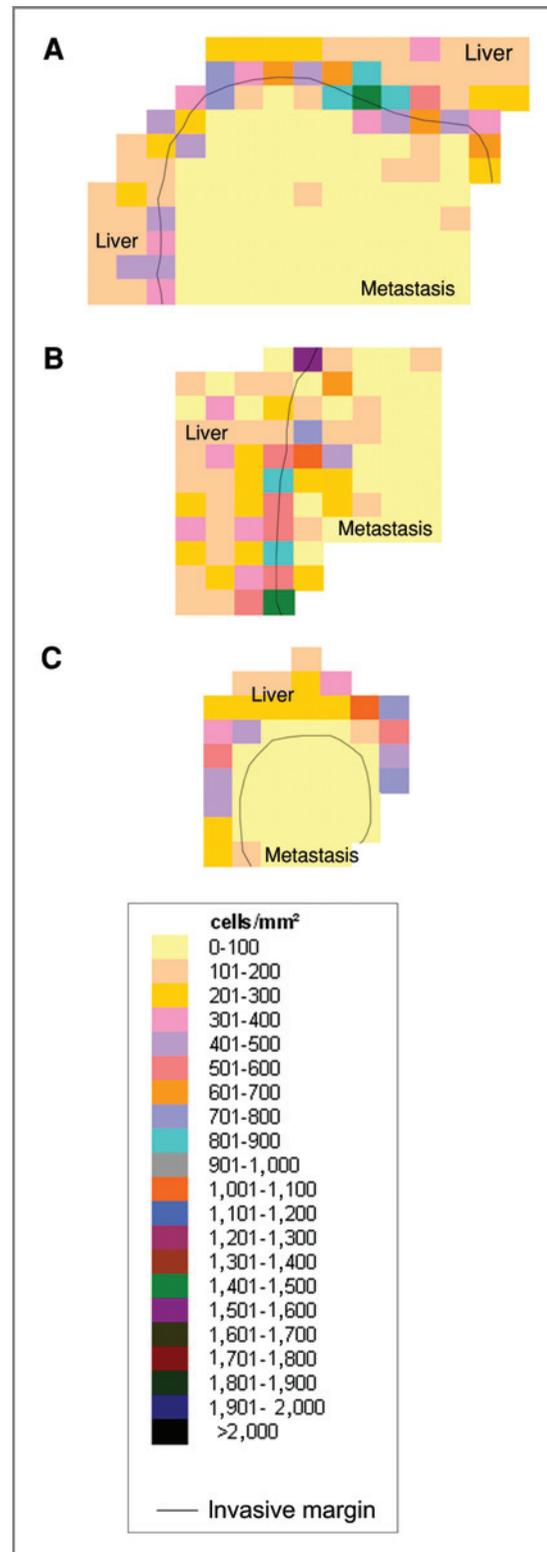


Figure 4. Immunomap of 3 independent CRC liver lesions. Complete tissue surface sections are displayed and artificially divided into tiles (each tile being 1 mm²) and subsequent quantification of CD3⁺ T cells for each tile. Each tile is then colored according to the T-cell density. The black line indicates the invasive margin.

biopsies then often do not include an invasive margin area. Surgical specimens, however, allow ample evaluation of tissue sections, especially with regard to invasive margins. This might be an explanation for the misclassified patient samples: a high rate of 7 needle biopsies of 10 samples among the misclassified patient samples. The observed heterogeneity of infiltrate density at the invasive margin within the same patient (see Fig. 4 for 3 different patient samples) might be the source for higher misclassification on smaller areas of invasive margin. Only whole slide quantification allows therefore to generate robust cell quantities along large stretches of invasive margin.

Previous work by others showed a prognostic significance for T-cell infiltrates at the invasive margin of the primary tumor underlining the importance of immune reactions (9, 13). The cutoff values for T cells (at the invasive margin of liver metastases) used for response prediction in this set of patients are similar to the cutoff values defined by previous work in primary tumors (9). The primary site of interaction between malignant cells and immune cells is the invasive margin in both primary tumors and in metastases, showing considerable variation in immune cell composition (see Fig. 4; ref. 16). This concordance in cutoff values points to an underlying general relation between proliferating cancer cells and an active immune response. So our observation in metastatic lesions supports the findings in the primary tumor with respect to the prognostic implication and represents the first detailed evaluation of TILs in partially removed metastatic liver lesions. A complete evaluation of the response to chemotherapy would of course require a comparative analysis including patients without chemotherapy, which is unethical in the metastatic setting. These observations, however, raise important questions about the treatment of patients and might help to identify metastatic CRC patients likely to benefit from chemotherapy.

The 2 cohorts included different patients with a range of various different chemotherapy and (immunologic) antibody treatment regimens and combinations. Nevertheless, the association between immune cell density and treatment outcome could be detected. As for the effects of chemotherapy on the immune system, 5-fluorouracil is included in all treatment regimens and can at least partially deplete or transiently inactivate inhibitory immune cells (25). Oxaliplatin induces immunogenic cell death (25) but in our investigation, it did not show clear superior effects compared with irinotecan. As different chemotherapeutic agents have different immunologic effects, they might as well have different "threshold levels" for the immune cell density to have a good prognostic impact, "threshold levels" that could not be delineated in the current study. For antibodies, the immunologic aspects are well recognized (25), but what is the effect of chemotherapy on immune cells? One important mechanism of chemotherapy might be the immunomodulation, that is, suppression of inhibitory immune cell function, allowing the surrounding T cells to infiltrate and attack cancer cells. This is a hypothesis that is in line with the cancer immunosurveillance theory developed by Dunn and colleagues (26–28) and Koebel and colleagues (29) which suggests that tumors are controlled by

the immune system (equilibrium phase) and eventually escape immunosurveillance (30). The precise elements of this immunosurveillance or immunoediting are unknown. In mouse models, the administration of chemotherapy could selectively deplete regulatory T cells while sparing effector T cells and enhancing dendritic cell function (31). Other studies indicate a possible role for regulatory T cells specifically in human CRC (32). As their numbers are very low in metastatic CRC liver metastases, they do not seem to have large impact in the metastatic situation. Recent data from CRC patients supported the idea that the complex network of inhibitory and activating immune signals seems to change during the course of the disease (33, 34) and there are of course other inhibitory (immune) cell populations that also have potent suppressive functions on effector T cells (e.g., myeloid-derived suppressor cells) that also could be affected by chemotherapy (35). In an expression profiling study on the invasive margin of CRC liver metastases by Lassmann and colleagues, mRNA levels of *cyclin D1* and *survivin*, 2 genes involved in cell proliferation and antiapoptosis, were associated with hepatic recurrences (36). As a hypothesis, these upregulated transcripts could also come from "resistant" inhibitory immune cell subpopulations, thereby effectively hindering the above-mentioned effects of chemotherapy on this subset of immune cells.

Our results show the distribution and detailed localization of T cells at the tumor site—and especially at the invasive margin of liver metastases of CRC. The observed densities at the liver metastases imply a similar impact of these cells as observed in primary CRC tumors. The presence of the infiltrates is linked with successful chemotherapy in metastatic CRC. This observation could have implications for the treatment of patients with advanced CRC. The analysis of immune responses at the site of metastatic disease might help to select patients likely to benefit from systemic treatment and on the other hand, prevent patients unlikely to respond from treatment-related side effects. These findings can aid in the development of a new staging system for advanced CRC (37). Taken together, the presented data will have implications for the assessment of treatment options for patients with metastatic CRC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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