Tumor Infiltration by T Lymphocytes Expressing Chemokine Receptor 7 (CCR7) Is Predictive of Favorable Outcome in Patients with Advanced Colorectal Carcinoma

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Introduction

Colorectal carcinoma (CRC) is the third most common cause of cancer worldwide (1). At the present, the best therapeutic option for advanced disease is represented by chemotherapy regimens containing fluorouracil (5-FU) ± levofolinic acid (LF) together with irinotecan (FOLFIRI) or oxaliplatin (FOLFOX), given alone or in combination with bevacizumab, a monoclonal antibody (mAb) to the vascular-endothelial growth factor, or cetuximab and panitumumab, mAbs directed against the epidermal growth factor-receptor (2). For patients with advanced disease there are no effective parameters able to support the choice of specific treatments, which, currently, are selected on the basis of risk factors, performance status, and more recently on the presence in the tumor tissue of activating k-ras mutations, which makes ineffective the addition of cetuximab or panitumumab to chemotherapy (3).

Recently, it has been proposed that adaptive antitumor immune response and autoimmunity in cancer patients, occurring spontaneously or as a consequence of specific treatments, may be a critical feature for good outcome and prolonged survival (4). On the basis of preclinical findings (5), we designed a novel therapeutic regimen that includes a mAb to chemotherapy (3).

Purpose: An efficient adaptive immunity is critical for a longer survival in cancer. We investigated the prognostic value of tumor infiltration by CD8+ T cells expressing the chemokine-receptor-7 (Tccr7) and the correlation between tumor infiltration by Tccr7 and regulatory CD4+ FoxP3+ T cells (Treg) in 76 metastatic colorectal cancer (mCRC) patients enrolled in a phase III trial.

Experimental Design: Tccr7 and Treg cell infiltration in tumor samples was quantified by immunohistochemistry. The correlation among Tccr7, Treg tumor infiltration, and patients’ outcome was evaluated.

Results: High Tccr7 tumor infiltration was predictive of prolonged OS [high vs. low Tccr7 score: median 38 months (95% CI: 24.5–51.4) vs. 20 months (95% CI: 11.4–28.5); HR = 0.48 (95% CI: 0.24–0.96); P = 0.03] and prolonged progression-free survival [PFS; high vs. low Tccr7 score: median 12 months (95% CI: 7.7–16.2) vs. 7 months (95% CI: 5.2–8.7); HR = 0.54 (95% CI: 0.28–1.01); P = 0.01] after front-line chemotherapy. Regression analysis did not show correlation between Tccr7 and Treg infiltration levels. However, the cluster of patients showing concomitant high infiltration by both Tccr7 and Treg disclosed a favorable outcome [double high vs. double low tumor infiltration score: median OS = 35 months (95% CI: 20.8–49.1) vs. 17 months (95% CI: 4.6–29.3); HR = 0.32 (95% CI: 0.12–0.87); P = 0.02] and median PFS = 11 months (95% CI: 9.4–12.5) vs. 5 months (95% CI: 2.2–7.7); HR = 0.43 (95% CI: 0.17–1.06); P = 0.01].

Conclusions: High Tccr7 tumor infiltration score is a favorable prognostic factor for mCRC. Our findings underline the relevance of microenvironment-related immunologic events for patient outcome. Clin Cancer Res; 18(3); 850–7. ©2011 AACR.
in the aim to activate peripheral dendritic cells (DC) and rIL-2 to endure an antitumor antigen-specific T-cell–mediated immune response. This chemo-immune-burst regimen, named GOLFIG, resulted safe and active in largely pretreated metastatic CRC (mCRC) patients in a previous phase II trial (7, 8). In that study, we registered in 19% of patients the occurrence of autoimmunity which resulted the most efficient predictor of good outcome in a multivariate analysis (8). Moreover, we observed significant changes in specific T-lymphocyte subsets both in the primary tumor and in the peripheral blood. Along the treatment, it was observed a progressive increase of tumor antigen [Carcinoembryonic antigen (CEA) and thymidylate synthase (TS)]-specific cytotoxic T-cell precursors and central memory T-lymphocytes (CD8+CD45RA-CCR7+) T cells expressing the chemokine receptor-7 (CCR7; Tcm) has a favorable prognostic value in these patients. We also report a correlation among tumor infiltration by both Tcm5 and Treg8 (CD4+FOXP3+) lymphocytes and patients’ outcome. These results provide novel information on the immunologic microenvironment of colorectal cancer and, therefore, offer the rationale for novel therapeutic approaches, which might take benefit from the antitumor immune response in the microenvironmental milieu, and put forward a new perspective to enhance the activity of colorectal cancer treatment.

**Translational Relevance**

Our report describes the results of a preplanned side study aimed to evaluate in a prospective setting the role of different tumor-infiltrating T-lymphocyte subsets at baseline in advanced colorectal cancer patients enrolled in the GOLFIG-2 phase III trial. This study was designed with the major aim to compare the antitumor activity of GOLFIG (gemcitabine + oxaliplatin, levo-folinic acid, and 5-FU followed by granulocyte macrophage colony-stimulating factor and recombinant interleukin-2) chemo-immune-burst versus the FOLFOX-4 (oxaliplatin, levo-folinic acid, and 5-FU) regimen, based on the promising results of a previous phase II study. We show that high tumor infiltration by cytotoxic (CD8+ T) cells expressing the chemokine receptor-7 (CCR7; Tcm) has a favorable prognostic value in these patients. We also report a correlation among tumor infiltration by both Tcm5 and Treg8 (CD4+FOXP3+) lymphocytes and patients’ outcome. These results provide novel information on the immunologic microenvironment of colorectal cancer and, therefore, offer the rationale for novel therapeutic approaches, which might take benefit from the antitumor immune response in the microenvironmental milieu, and put forward a new perspective to enhance the activity of colorectal cancer treatment.

**Patients and Methods**

**Patient characteristics**

The inclusion criteria were: written informed consent, histologically confirmed diagnosis of CRC, no previous chemotherapy for advanced disease, measurable disease (according to WHO tumor response criteria), an ECOG performance status ≤ 2, normal renal and hepatic function, white blood cell count 2,500/mm3 or more, hemoglobin levels 9 g/dL or more, platelet cell count 100,000/mm3 or more and normal cardiac function. The exclusion criteria were: any major organ failure, central nervous system involvement, second malignancies, active infectious disease, major autoimmune diseases, and acquired immune suppression.

**Treatment schedules and patients’ evaluation**

All patients were randomized to receive FOLFOX-4 or GOLFIG treatment. The FOLFOX-4 arm received biweekly chemotherapy with oxaliplatin (85 mg/m2 on day 1), LF (100 mg/m2 on days 1–2) and 5-FU (400 mg/m2 as a bolus and 600 mg/m2 as a 22 hour infusion on days 1–2); the GOLFIG arm received biweekly chemotherapy with gemcitabine (1,000 mg/m2 on days 1 and 15), oxaliplatin (85 mg/m2 on days 2 and 16), LF (100 mg/m2 on days 1,2,15 and 16), and 5-FU (400 mg/m2 as a bolus and 800 mg/m2 as a 24-hour infusion on days 1, 2, 15, and 16), followed by...
assessed the rationale for the trial was to compare efficacy of GOLFIG chemotherapy with FOLFOX-4 chemotherapy as frontline treatment for mCRC patients. Time period for enrolment was from 2005 July to 2010 November. Median follow-up was 18 months. The established observation period from the end of enrolment was estimated of 3 years, due to the prognosis in advanced colorectal disease. Primary end point was a 2 months advantage in terms of PFS in the GOLFIG arm, and secondary endpoints were OS and response rate (RR).

### Statistical analysis

We carried out a preplanned immuno-histochemistry analysis on specimens collected from 76 patients consecutively enrolled in GOLFIG-2 trial and randomized to receive FOLFOX-4 chemotherapy or GOLFIG chemotherapy. In particular, we investigated the possible prognostic value of tumor infiltration by several lymphocyte subsets [CD4⁺, CD8⁺ (CTLs), TCCR7 (CD8⁺CCR7⁺), and Treg (CD4⁺FoxP3⁺)] on the surgical pathology sample. At this aim, OS and PFS were evaluated with the Kaplan-Meyer, whereas log-rank test was carried out to evaluate statistical differences between the groups with high and low TCCR tumor infiltration score method (SPSS 17 statistic software). A post-hoc multivariate analysis through COX's regression analysis (NCSS statistical software), with several prognostic factors at trial entry (performance status; sex; age; tumor grading presence or absence of liver metastases) was also carried out to assess the independent prognostic value of each variable. Regression analysis (by the GraphPad Instat 3.2 statistic software) was carried out to evaluate whether the above mentioned survival parameters were significantly

### Specimen characteristics

**Pathology study.** Tumor tissues derived from biopsy or radical surgery were fixed in 10% buffered neutral formalin and paraffin embedded for histology and immunohistochemistry. Sections of each specimen were stained with hematoxilin and eosin and histologically examined by an expert pathologist.

**Assay methods**

**Immunohistochemistry.** Immunohistochemical staining was carried out on 3-μm thick sections of each block by the streptavidin-biotin method. The cores were taken randomly from within the tumor block face and at least 3 different samples for each patient were evaluated. After being dewaxed and rehydrated, sections were incubated with 3% H₂O₂ in TBS solution to inhibit endogenous peroxidase and processed with different methods for each antibody. To show CD4⁺ T cells, the sections were unmasked with Wcap buffer (pH 6.0 for 40 minutes at 98°C; Bio-Optica) and were incubated with anti-human monoclonal antibody CD4 (clone 4B12; 1:50; Menarini). For CD8⁺ T cells, pretreatment with a microwave oven in citrate buffer (0.01 mol/L, pH 6.0) at 750 W for 5 minutes was carried out for 3 cycles, and the sections were incubated with anti-human monoclonal antibody CD8 (clone CD8-144B; 1:50; Dako); the epitopes were detected with the Ultravision Detection System and revealed with the diaminobenzidine for 5 minutes (Dako). For detection of FoxP3⁺T cells, we used EDTA (0.05 mol/L, pH 8.0) pre-treatment in a microwave oven at 750 W for 5 minutes. After 3 cycles, the sections were incubated with anti-human FoxP3 mAb (clone 22510; 1:50; 60 minutes; Abcam) or CCR7 mAb (clone 150503 MAb 197; 1:20; 60 minutes; R&D Systems).

The primary antibody enhancer was associated with an AP Polymer (Ultravision LP Detection System AP Polymer, LAB Vision) and revealed with fuchsin (DakoCytomation). Negative controls were obtained by replacing the specific antibody with nonimmune serum immune-globulins at the same concentration of the primary antibody. Immune staining was examined with a Zeiss Axiosplan 2 microscope (Carl Zeiss Microscopy). Blind reanalysis was carried out to confirm the results.

**Quantitative evaluation of T-lymphocyte subtypes**

Sections stained for CD4⁺, CD8⁺, CCR7⁺, or FoxP3⁺-lymphocytes were scored in coded slides by 1 observer. The number of positive cells for each marker was recorded in 5 randomly chosen HGPFs in the stroma adjacent to neoplastic glands. Cut-off value to discriminate between high and low lymphocyte infiltration was determined as 20 positive cells both for CCR7⁺- or FoxP3⁺-lymphocytes (median value of both groups). Actuarial survival curves and Cox analysis were constructed on the above described survival endpoints (PFS and OS). Regression analysis was also carried out to evaluate the potential correlation among the specific lymphocyte infiltration scores (as a continuous variable) and the survival endpoints.

### GOLFIG-2 study design

The current study has been designed as a preplanned biological analysis in the framework of the GOLFIG-2 prospective clinical trial. The phase III trial was authorized by the Institutional Ethical Committee and by the Italian Ministry of Health. The role of the steering Committee was to provide executive oversight and supervision for the conduct of the trial, through review of trial enrolment, protocol and clinical conduct, and blinded safety data. All patients were in first line chemotherapy for mCRC and signed an informed consent.

The rationale for the trial was to compare efficacy of GOLFIG chemotherapy with FOLFOX-4 chemotherapy as frontline treatment for mCRC patients. Time period for enrolment was from 2005 July to 2010 November. Median follow-up was 18 months. The established observation period from the end of enrolment was estimated of 3 years, due to the prognosis in advanced colorectal disease. Primary end point was a 2 months advantage in term of PFS in the GOLFIG arm, and secondary endpoints were OS and response rate (RR).
correlated to $T_{ccr7}$ tumor infiltration extent as a continuous variable. Unpaired 2-tailed $t$ student test (by the GraphPad Instat 3.2 statistic software) was carried out to compare survival means between different groups.

Results

Demography and study design

Seventy-six patients with mCRC, 48 males and 28 females, with a median age of 65 years were included in this study. These patients had been consecutively enrolled in the GOLFIG phase III trial and randomized to receive upfront treatment according to FOLFIR-4 chemotherapy (44 patients) or GOLFIG (32 patients) chemoimmunotherapy.

An immunohistochemistry study, performed to evaluate the extent of tumor infiltration by $T_{ccr7}$ and $T_{reg}$ was carried out on tumor samples obtained from these patients before any systemic treatment. This analysis included fewer patients in the GOLFIG treatment arm because for many of them there were not adequate histologic samples available for the immunohistochemistry study.

For the correlative studies, the high and low lymphocyte expression levels were calculated on the basis of the median value of the number of lymphocytes expressing the specific marker (CD8 for CTLs and CCR7 for $T_{ccr7}$) detected in each high power slide field (Fig. 1A–E). The characterization of FoxP3$^+$ T cells as active $T_{reg}$ with immunosuppressive status was defined in a previous study (9). A double color immunohistochemistry characterization carried out on 10 samples showed CCR7$^+$ expression mainly on CD8$^+$ T cells (Fig. 1F).

High expression of CCR7-positive TILs is predictive of good outcome in patients with advanced CRC

Our analysis indicated that a high tumor $T_{ccr7}$ infiltration score at the diagnosis correlated with a prolonged OS [high vs. low $T_{ccr7}$ score: median 38 months (95% CI: 24.5–51.4) vs. 20 months (95% CI: 11.4–28.5); HR = 0.48 (95% CI: 0.24–0.96); $P = 0.03$] and prolonged PFS [high vs. low $T_{ccr7}$ score: median 12 months (95% CI: 7.7–16.2) vs. 7 months (95% CI: 5.2–8.7); HR = 0.54 (95% CI: 0.28–1.01); $P = 0.01$] after front-line chemotherapy. The latter result provides clear evidence that $T_{ccr7}$ has a prognostic value in mCRC patients (Fig. 2A–B).

A further multivariate COX analysis also indicated a favorable prognostic value for $T_{ccr7}$ infiltration score in terms of prolonged OS ($P = 0.05$). Moreover, a linear regression analysis confirmed the statistical correlation between PFS and tumor infiltration by $T_{ccr7}$ cells as a continuous variable ($r^2 = 0.11; P = 0.02$; Fig. 3A), paralleled by a $t$ test, carried out by grouping the patients considering $T_{ccr7}$ values as a dicotomic high/low variable and comparing the mean PFS values (low CCR7 = 7.7 months, high CCR7 = 17.3 months, $P = 0.02$; Fig. 3B).

Correlation between $T_{reg}$ and $T_{ccr7}$ infiltration in tumor tissues

We next investigated for a possible correlation in term of tumor infiltration levels among different T-lymphocyte subsets. We were unable to show any correlation among $T_{ccr7}$ infiltration and: (i) total lymphocyte density; (ii) CD8$^+$/CD4$^+$ T-cell tumor infiltration; (iii) tumor grading; and (iv) tumor stage at the diagnosis (data not shown).

Regression analysis failed to show any significant correlation between $T_{ccr7}$ and $T_{reg}$ tumor infiltration extent. However, we identified 4 different clusters of patients, whose primary tumor presented infiltration by each one of these T-cell subsets according to the following scenario: (i) low $T_{reg}$ and low $T_{ccr7}$ (LL); (ii) high $T_{ccr7}$ and low $T_{reg}$ (HL); (iii) low $T_{ccr7}$ and high $T_{reg}$ (LH); high $T_{reg}$ and high $T_{ccr7}$ (HH), which were associated with different outcomes. In fact, we detected the longest survival in those patients who showed a combined high tumor $T_{reg}$ and $T_{ccr7}$ infiltration and the worst outcome in those who presented a combined tumor infiltration by either lymphocyte subsets [double high vs. double low tumor infiltration score: median OS = 35 months (95% CI: 20.8–49.1) vs. 17 months.
(95% CI: 4.6–29.3); HR = 0.32 (95% CI: 0.12–0.87); P = 0.02, with more than 20% patients surviving more than 75 months, and median PFS = 11 months (95% CI: 9.4–12.5) vs. 5 months (95% CI: 2.2–7.7); HR = 0.43 (95% CI: 0.17–1.06); P = 0.01; Fig. 4A–B).

These analyses were carried out on the whole patient population and did not take in account the actual patient treatment; it was, in fact, not possible to achieve any significant results in either the FOLFOX-4 or GOLFIG arm due to the small sample and this important investigation point was considered beyond the scope of the present report.

Discussion

The results of the present study suggest that a greater infiltration by CD8\(^+\)CCR7\(^+\) T cells in the primary tumor at the diagnosis is predictive of better outcome in advanced mCRC patients. We were unable to show a direct statistical correlation between T\(_{\text{CCR7}}\) and T\(_{\text{reg}}\) tumor infiltration extent; however, a concomitant high expression of both T-lymphocyte subsets in the tumor helps to identify a cluster of good prognosis patients. In contrast, those patients, whose tumor tissue presents a combined low infiltration score for both lymphocyte populations, have a very poor outcome. These findings withstand the hypothesis that primary tumor infiltration by different T-cell subsets may reflect the state of immune-competence of the patients and produce a reliable prognostic factor. A limit of the present study is the lack of a confirmatory data set. To this end, we are planning confirmatory analysis within the whole trial patient population and, in additional series, to provide additional evidence on the prognostic role of T\(_{\text{CCR7}}\) lymphocytes in colorectal cancer as shown in this hypothesis-generating study.

Zitvogel and colleagues (4) has already highlighted the important role played by the immune-surveillance in conditioning cancer patient survival. These authors hypothesized a possible synergistic interaction between administration of cytotoxic drugs and host immune response. They have in fact speculated that chemotherapy is able to reduce the immunosuppressive tumor burden (4, 21), induce antigen remodeling (immune-editing), and promote immunologic danger signals (5, 22). Moreover, cytotoxic drug treatment may also damage immunosuppressive cell...
populations, such as inhibitory myeloid cells or T<sub>reg</sub>, which have been often found mostly overexpressed in cancer patients (4).

Several studies in mCRC patients have also investigated the prognostic role of the basal inflammatory status and lymphocyte density in the primary tumor with conflicting results (23–26). A reliable prognostic value has been proposed for tumor infiltration by professional antigen presenting cells such as macrophages and DCs in patients who underwent surgery for CRC (27).

A more controversial scenario concerns the prognostic role of tumor infiltration by T<sub>reg</sub> cells defined by a CD4<sup>+</sup>CD25<sup>hi</sup>FoxP3<sup>+</sup> immune-phenotype in CRC patients. This lymphocyte subset, which has a powerful immunosuppressive activity, is often overexpressed during viral infection, chronic inflammatory diseases, and cancer, in the attempt to prevent a dangerous overresponse and autoimmunity (28–31). Recently, it has been shown that tumor infiltration by immune-regulatory T cells defined by a T<sub>reg</sub> immune-phenotype is a favorable prognostic factor in early and mCRC. Salama and colleagues, in fact, showed, on a very large series of early CRC patients, that T<sub>reg</sub> infiltration in the primary tumor is predictive of longer survival. They also showed that the protective value was completely lost when the T<sub>reg</sub> presence was detected in the draining nodes (23).

Similarly, our group has shown that high T<sub>reg</sub> tumor infiltration in mCRC patients enrolled in the GOLFIG-2 phase III trial is predictive of favorable outcome in term of OS and PFS (9). The findings from this early report on T<sub>reg</sub> and the current study on T<sub>CCR7</sub> infiltration, and specifically the data on double positive (high T<sub>reg</sub> and high CCR7<sup>+</sup>) versus double negative (low T<sub>reg</sub> and low CCR7<sup>+</sup>) depict a scenario in which different lymphocyte populations may represent marker of protective anticancer response which has an important impact on patient outcome.

Figure 4. Actuarial Kaplan-Meyer’s survival curves of patients whose primary tumor presented a high infiltration score of both T<sub>CCR7</sub> and T<sub>reg</sub> subsets (— — — —) compared with those presenting a double low infiltration score (———) of either lymphocyte populations. Double T-cell population infiltration score was respectively compared in term of: (A) OS and (B) PFS.
We can also take in consideration that CD3\(^{+}\)CD8\(^{+}\) (including T\(_{c\alpha7}\)) T lymphocytes, in comparison with T\(_{reg}\) are much more resistant to chemotherapy, as we showed in a previous study (22). These lymphocytes are, in fact, sensitive to the cytotoxic effects of chemotherapy only when they are in active proliferation, which takes place in response to antigen presentation by DCs and by other antigen presenting cells in the primary lymphoid organs only. On this basis, T\(_{c\alpha7}\) lymphocytes are mostly refractory to the drugs after their expansion and release from the primary lymphoid organs. On the other hand, T\(_{reg}\) proliferation may occur in situ where it is nourished by selective cytokine/chemokine production in the tumor microenvironment. Several studies have already shown that T\(_{reg}\) are sensitive to cytotoxic drugs such as cyclophosphamide (36, 37), gemcitabine, 5-FU, or oxaliplatin (22).

In conclusions, the results of this study indicate that T\(_{c\alpha7}\) infiltration is a favorable prognostic factor for mCRC patients and suggest that high T\(_{reg}\) infiltration represents a feedback to a preexisting immune response. We believe that our findings on the immunologic tumor microenvironment hearten additional investigation on this specific topic to gain relevant information for the design of more efficient therapeutic strategies for mCRC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

This study has been supported by a grant from the Italian Ministry of Scientific Research (MIUR). Unit Leader: Prof. Maria Teresa Del Vecchio; Project Coordinator: Prof. Angelo Aquino.

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Received February 12, 2011; revised November 20, 2011; accepted November 25, 2011; published OnlineFirst December 5, 2011.

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