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 chemotherapy. The correlation among Tccr7, Treg tumor infiltration, and patients' outcome was evaluated. 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 16.2) vs. 7 months (95% CI: 5.2–8.7); HR = 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+) together with a progressive reduction of immune suppressive regulatory T cells (CD4+CD25hiFoxP3+; Treg; refs. 7, 8). These intriguing results led us to design a phase III trial, which is presently ongoing in mCRC patients (GOLFIG-2 trial). This study was aimed to evaluate the efficacy of front line GOLFIG regimen in comparison with the standard FOLFOX-4 chemotherapy.

We also preplanned an immune biological investigation, aimed to evaluate in a prospective setting the role of different tumor-infiltrating T-lymphocyte subsets at baseline in mCRC patients enrolled in the GOLFIG-2 trial. In this context, a first analysis revealed a highly favorable outcome in those patients whose primary tumor at diagnosis was associated with high infiltration by lymphocytes expressing Treg immunophenotype. A high Treg tumor infiltration score was strongly predictive of prolonged overall survival (OS) and progression-free survival (PFS) in either FOLFOX-4 or GOLFIG treatment arms (9). Considering that this lymphocyte subset represents an inhibitory feed-back response to a preexisting immune stimulation (10–15), we formulated the hypothesis that Treg infiltration in the tumor tissue might be an indirect and powerful indicator of local antitumor immune response. On these bases, we have subsequently investigated whether Treg infiltration is also correlated to the presence of other activated immune effectors. In particular, we evaluated in the same patient population whether the level of tumor infiltration by CD8+ T cells expressing CCR7 could predict patient outcome and might correlate with the extent of Treg tumor infiltration.

In this study, we focused therefore on the population of lymphocytes expressing a CD8+CCR7+ phenotype, including naïve (CD8+CD45RA+CCR7+), central memory (CD8+CD45RA−CCR7+CD27+CD62L−; Tcm) subsets, which represent a fresh source of activated immune effectors. These cells under specific microenvironmental conditions may either differentiate in antigen-specific long-term memory or highly cytotoxic T-cell effectors (16, 17). We decided to investigate lymphocytes bearing CCR7 (TCCR7), taking in account that it is a homing receptor for chemokine ligand 19 and 21 (CCL19, CCL21; ref. 18), which are able to drive these effector lymphocytes in lymph nodes and in sites were the immune attack takes place (19). The manuscript has been drafted according to REMARK criteria (20).

**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...
subcutaneous rGM-CSF (100 µg, on days 3–7) and ultra low-dose subcutaneous rIL-2 (0.5 × 10^6 IU s twice a day on days 8–14 and 17–29; ref. 7). Standard assessments (clinical history, physical examination, blood chemistry, evaluation of serum CEA, and CA19.9 concentration) were carried out at baseline and repeated every 2 weeks, chest x-ray and ultrasound scans every 4 weeks. High-definition, multislice computed tomography scans with contrast medium were recorded every 3 months. Both treatments were continued until disease progression, occurrence of unacceptable toxicity, clinical judgment, or withdrawal of consent. All patients were evaluated for PFS (calculated from trial enrolment to disease progression or death) and OS (calculated from trial enrolment to death).

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 from each biopsy sample for each patient. Positive control sections were tested for background and specificity. For all initial and follow-up immunohistochemical analysis, 10 slides were stained per patient. The slides were prepared using an automated staining system (UltraVision Detection System, Lab Vision) 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) pretreatment in a microwave oven at 750 W for 5 minutes. After 3 cycles, the sections 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) pretreatment 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; Abcam) or CCR7 mAb (clone 150503 MAb 197; 1:20; 60 minutes; Abcam) or CCR7 mAb (clone 150503 MAb 197; 1:20; 60 minutes; Abcam) or CCR7 mAb (clone 150503 MAb 197; 1:20; 60 minutes; Abcam) or CCR7 mAb (clone 150503 MAb 197; 1:20; 60 minutes; Abcam) or CCR7 mAb (clone 150503 MAb 197; 1:20; 60 minutes; Abcam) or CCR7 mAb (clone 150503 MAb 197; 1:20; 60 minutes; Abcam) or CCR7 mAb (clone 150503 MAb 197; 1:20; 60 minutes; Abcam). For FoxP3+ T cells, the sections were incubated with anti-human monoclonal antibody CD4 (clone CD4–144B; 1:50; Menarini). For CD8+ T cells, the sections were stained for CD4+ CCR7−, CD8− FoxP3−, and CD8+ CCR7− FoxP3−. Negative controls were obtained by replacing the specific antibody with nonimmune serum. High-definition, multislice computed tomography scans with contrast medium were recorded every 4 weeks. High-definition, multislice computed tomography scans with contrast medium were recorded every 3 months. Both treatments were continued until disease progression, occurrence of unacceptable toxicity, clinical judgment, or withdrawal of consent. All patients were evaluated for PFS (calculated from trial enrolment to disease progression or death) and OS (calculated from trial enrolment to death).

GOLFIG-2 study design
The current study was designed as a preplanned biological analysis in the framework of the GOLFIG-2 prospective clinical trial. The phase III trial was authorized 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.

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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 chemoimmunotherapy. In particular, we investigated the possible prognostic value of tumor infiltration by several lymphocyte subsets [CD4+ CCR7−, CD8+ CCR7−, and FoxP3+ T cells]). The rationale for the trial was to compare efficacy of GOLFIG chemoimmunotherapy 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).

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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 FOLFOX-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 \( (r^2 = 0.11; P = 0.02; \text{Fig. 3A}) \), 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_{CCR7} and T_{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_{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 GOLF-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.

T<sub>ccr7</sub> lymphocytes population includes 2 different populations of CD8<sup>+</sup>CCR7<sup>+</sup> T immune-effectors which can be distinguished in naive and central memory T cells depending on CD45RA expression. These lymphocytes are recalled by specific chemokines in the tumor site, where eventually differentiate in antigen specific T cells with long-term memory cells or effector cells able to kill the specific tumor targets with high efficiency (16). CCR7 is a receptor able to bind different ligands (CCL-19 and 21) produced by activated DCs and other inflammatory cells (32, 33). Once engaged by its ligands during the immune attack, CCR7 regulates the homeostatic recirculation through body cavities and primes an intracellular process in the T cells that guides their chemotactic homing to lymph nodes, tumor/infected tissues and target cells (34). These lymphocytes also acquire the expression of CD62L, an adhesion molecule that plays a primary role in T-cell homing by mediating leukocyte interaction with activated vascular endothelium in high endothelial venules (16, 33, 34).

An increase in the number of T cells expressing these receptors indicates a greater amount of freshly mobilized lymphocytes available to differentiate in immune-effector that sustain a more prolonged antigen-specific T-cell–mediated immune response.

The results of a large pathology study, supported by microarray transcriptional profiling of early colorectal cancer samples, revealed that the presence of memory T lymphocyte infiltration and activated cytotoxic T-cell gene signature is strongly predictive of prolonged survival and reduced risk of relapse, while no prognostic role for inflammatory microenvironment was shown (35). The same authors confirmed in these patients with early CRC an adverse prognostic value for high vascular, lymphatic, and perineural tumor cell infiltration score (VeLiPi), reporting that it was inversely correlated with memory T-cell tumor infiltration and memory molecular profiling signature.
We can also take in consideration that CD3+CD8+ (including T_{CCR7}) 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_{CCR7} 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_{CCR7} infiltration is a favorable prognostic factor for mCRC patients and suggest that high T_{reg} infiltration represents a feed-back 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.

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38. Prognostic Value of Tumor Infiltrating CCR7+ T Lymphocytes
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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