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High prevalence of Foxp3 and IL-17 in MMR-proficient colorectal carcinomas.

Sabine Le Gouvello [1,6,7], Sylvie Bastuji-Garin [2,6], Nijez Aloulou [4], Hicham Mansour [1], Marie-Thérèse Chaumette [3,6], François Berrehar [1], Amal Seikour [3], Antoine Charachon [4,6], Mehdi Karoui [5,6], Karen Leroy [3,6], Jean-Pierre Farcet [1,6], and Iradj Sobhani [4,6].

From the AP-HP, Hôpital Henri Mondor, Departments of Biological Immunology[1], Public Health[2], Pathology[3], Gastroenterology[4] and Surgery[5], and University Paris XII[6]; and the INSERM U841[7], 94010 Créteil, France.

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Short Title: Foxp3 and IL-17 overexpression in colon carcinomas

Corresponding author:
Iradj Sobhani (department of Gastroenterology)
Hôpital Henri Mondor
51 av. du Maréchal de Lattre de Tassigny
94010 Créteil - France
Tel: (+33) 1 49 81 23 62 or 2664
Fax: (+33) 1 49 81 23 52 or 2897
Email: iradj.sobhani@hmn.aphp.fr & sabine.le-gouvello@hmn.aphp.paris.fr

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Abbreviations:
MMR : mismatch repair; MSI :microsatellite instability; MSS microsatllite stability
CRC : colon and rectal cancer; T : tumoral tissue; NT:no tumoral tissu ; qT-PCR and RT-PCR : real time quantification of mRNA by PCR procedure; Treg :regulatory T cells ; Foxp3 : forkhead transcription factor ; FasL : Fas ligand ; Gz : Granzyme

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Competing Interest
None to declare.

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Abstract

Background & Aims: Colon cancer (CRC) harbors different types of DNA alterations, including microsatellite instability (MSI). Cancers with high levels of MSI (MSI-H) are considered to have a good prognosis, probably related to lymphocyte infiltration within tumors. Our aim was to characterize the intratumoral expression of markers associated with the anti-tumour immune response in MMR-proficient (MSS) colon cancers.

Methods: Ninety human colon cancers (T) and autologous normal colon mucosa (NT) were quantified for the expression of 15 markers of the immune response with RT-PCR. mRNA levels were correlated with MMR status. Immunohistochemistry (IHC) including both IL17 and CD3 antibodies was used.

Results: Expression of cytotoxic markers (FasL, granzyme B and perforin), inflammatory cytokines (IL-1β, IL-6, IL-8, IL-17 and TGFβ), and a marker of regulatory T cells (Foxp3) were significantly higher in tumors than in autologous normal tissues. Adjusting for MMR status, higher tumoral expression of both Gz B and perforin was associated with the MSI-H phenotype, and the perforin T/NT ratio was higher in MSI-H tissues than in MSS tissues. Higher tumoral expression of Foxp3, IL-17, IL-1β, IL-6, and TGF-β was associated with the MSS phenotype, and the IL-17 T/NT ratio was higher in MSS tissues than in MSI-H tissues by using either RT-PCR or IHC.

Conclusions: Immune gene expression profiling in CRC displayed different patterns according to MMR status**.** Higher Foxp3, IL-6, TGF-β and IL17 expressions are particular determinants in MMR-proficient CRC. They may be potential biomarkers for a new prognostic "test set" in sporadic CRCs.

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Introduction

Colorectal cancer (CRC) is the second leading cause of cancer mortality in the Western world, despite recent advances in surgery, radiotherapy and chemotherapy{(1). Tumor progression of CRCs is governed by either genetic or epigenetic changes intrinsic to cancer cells, and by environmental factors. Approximately 15-20% of sporadic colorectal cancers and nearly all large bowel malignancies in the **Hereditary Non-Polyposis Colorectal Cancer** (HNPCC) syndrome are characterized by widespread microsatellite instability{(2), (3;4). Microsatellite instability is a genome wide instability in repetitive DNA sequences observed at the nucleotide level, and it is caused by the inactivation or loss of expression of the mismatch repair (MMR) genes: hMSH2, hMLH1, hPMS1, hPMS2, hMSH6/GTBP or MSH3, either as a result of mutations or epigenetic silencing{(5). Besides MMR deficient (MSI-H) cancer, the majority of sporadic colorectal cancers are MMR proficient (MSS) tumors with chromosomal instability in some cases{(6;7).

Most studies revealed longer disease-free and overall survival for patients with MSI-H CRC than for patients with MSS CRC (8-12)}, even in the case of deep local invasion of the primary tumor{(13)}. These studies suggested a protective role for T lymphocytes, which clearly infiltrated more extensively in MSI-H than in MSS CRCs{(14)}. Pronounced lymphocytic infiltration of tumors has long been associated with an improved prognosis{(15), (16)}. MSI in combination with a high content of intraepithelial cytotoxic lymphocytes was related to improved overall survival in a group of exclusively right sided CRCs {(17), (18)}, suggesting that a combination of both the tumor's local lymphocytosis and MMR characterization may be useful for a more accurate prognostic assessment. Contradictory results come from mouse models of

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colorectal cancer, and these results are consistent with the association between either chronic inflammation{(19;20)} or an increased number of inflammatory cells in tumors{(21) and tumor progression.

To determine the putative impact of microsatellite status on the anti-tumoral immune response, we analyzed histological and molecular features of tumors in patients with sporadic CRCs. We also developed a quantitative PCR assay for 15 inflammatory and T lymphocyte markers in tumours with reference to the MMR status.

Patients and Methods

Study population

This retrospective analysis included a group of 45 patients from a consecutive cohort survey study in Henri Mondor hospital. To be eligible, patients had to have undergone surgery of primary rectal or colon tumors, for which both frozen and paraffin embedded tumoral and normal tissues were available in the tissue collection bank. None had known hereditary cancer, ulcerative colitis, or Crohn's disease. Consecutive patients were routinely informed about biological researches on tissues that did not include hereditary genetic characterization. Their tissue materials could be used for the current study except if "formal opposition" was mentioned to doctors. Ethical committee approved the procedure.

Histology and pathology

Tissues were immediately examined after surgery, and normal and tumoral representative samples were frozen, fixed in formalin, and archived in a tissue collection bank. Diagnoses and tumor descriptions were done on H&S stained tissues according to the pTNM classification. Histopathology was further examined for lymphocyte infiltration and for a mucous component.

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Analysis of MMR status by immunohistochemistry

Representative samples from adenocarcinoma and normal mucosa adjacent to the tumor were selected in each case and paraffin sections were sent to an automated immunostainer. Tissue sections were incubated in citrate buffer with the following antibodies: G168-728 Ab (recognizes hMLH1 antigen, Ab was diluted 1:40; Pharmingen, San Diego CA-USA), FE11 Ab (recognizes hMSH2 antigen, Ab diluted 1:25; Calbiochem, Cambridge, USA), 44 Ab (recognizes hMSH6, Ab diluted 1:100; Zymed Laboratories Inc, South San Francisco, CA, USA), all in a boiling water bath. An avidin-biotin complex (ABC Vectastain Kit, Vector Lab) was used to reveal the antigen. Positive controls included slides from tumors with normal expression of hMLH1, hMSH2 and hMSH6; negative controls included slides with no primary antibodies. Two observers with no prior knowledge of the PCR results assessed all cases independently, and cases with discrepancies were further evaluated until agreement was reached between the observers.

Analysis of IL-17 expression by Immunohistochemistry

Representative samples (N>5) from tumoral and autologous normal mucosa were selected for each case, and paraffin-embedded sections (4µm) were treated by boiling in citrate buffer (pH 6) in a microwave (800W, 5min X3). Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide for 20 min followed by a wash in PBS for 5 min. Non-specific antibody binding sites were coated by the addition of a horse serum (1:20 in PBS, for 25 min). Serum was then removed and anti human IL17 goat antibody (R&D Systems, Lille, France) diluted in the PBS (1:40) was added to the tissue sections (room temperature for 1 h). Immunohistochemical staining was undertaken using a Vectastain Universal Elite kit according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA, USA). The chromagen, Sigmafast DAB (3′,3′-diaminobenzidine) (Sigma-Aldrich) was incubated with the tissue sections in the dark (room temperature for 4 min). The sections were the counterstained with haematoxylin.

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For double staining IL17/CD3, the goat anti-human IL-17 antibody (diluted 1:40) was applied first and incubated for 2h hrs. Immunohistochemical staining was undertaken using a Vectastain AP kit according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA, USA) and visualization was done with Naphtol/Fast Red(Sigma-Aldrich). Subsequently, a rabbit anti-human CD3 (diluted 1:50 in PBS, Dako, France) was incubated for 1 hr. Immunohistochemical staining was undertaken using ImmPRESS system (Vector Laboratories, Burlingame, CA, USA) and visualization was done with DAB substrate.

Analysis of microsatellite stability by PCR

Formalin fixed paraffin embedded (FFPE) sections from tumors were stained with hematoxylin and erythrosine B, and were macro-dissected if necessary to select a tissue fragment comprising more than 40% tumor cells. Normal and tumor autologous DNA was extracted from a 50µm FFPE or frozen tissue section with QiAmp DNA mini kits (Qiagen, Courtaboeuf, France), according to the manufacturer's instructions. The genetic instability (MSI-H) status of the tumors was established using pentaplex PCR for markers as described by Suraweera *et al.*{(22)}. Microsatellites were co-amplified in a single 20 µl pentaplex fluorescent PCR reaction, containing 0.1µM Bat-25, 0.25µM Bat-26 and NR-22, 0.5µM NR-24 and NR-21 primers, 0.15mM dNTP, 1.5mM $MgCl_2$, 1X GeneAmp PCR Buffer II (Applied Biosystems, Courtaboeuf, France), 50 ng genomic DNA and 0.5U AmpliTaq Gold DNA polymerase (Applied Biosystems). The PCR program was denaturation (95°C, 10 min), then 35 amplification cycles [denaturation (95°C, 30 sec.), annealing (55°C, 45 sec.), and extension (72°C, 30 sec.)], and a final elongation step (72°C, 7 min). Separation and detection of the fluorescent PCR products were performed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Courtaboeuf, France), and the data were analyzed with GeneScan Analysis Software (Applied Biosystems). The MSI-H phenotype was defined as the detection of length alterations of at least 3 markers in tumoral *vs* normal DNAs from the same

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individual .{ (22)}. We found that 31 / 45 tumoral tissues had the MSS phenotype, and 14 / 45 had the MSI-H phenotype.

Quantitative RT-PCR

Total RNA was prepared from 90 colorectal tumoral or autologous non-tumoral specimens, using Trizol® reagent (Gibco BRL, France) following the manufacturer's protocol. To avoid misinterpretation of the variability in marker gene expression due to heterogeneity of the cellular distribution and/or leukocyte densities in tissue samples, 10 sections of 50µ for each sample were verified before extracting total RNA. Microdissection was used if necessary to select tumour sections with tumoral cells higher than 60% of total. We normalized the ratio of mRNA copy number of the gene of interest / CD3 mRNA for each sample. First-strand cDNA was synthesized in RT samples, each containing: 2µg total RNA isolated from colorectal tissue, 16 U/µL M-MLV reverse transcriptase (Gibco-BRL, Life Technologies, Cergy-Pontoise, France), 4µM Oligo-(dT) 12-18 (Amersham-Pharmacia Biotech, Saclay, France) and 0.8 mM mixed dNTP (Amersham-Pharmacia Biotech, Saclay, France). Quantitative PCR was performed in a LightCycler 2.0 System (Roche Diagnostics, Meylan, France) using a SYBR Green PCR kit or a Hybridization Probes PCR Kit from Roche Diagnostics (Meylan, France), as previously described (23;24). The sequences of primers and probes are indicated in supplementary data. Normalization was achieved by quantification of the expression of the control housekeeping gene (HKG) β2µglobulin, which was chosen as the control among 3 HKG tested because of its stable expression in tumoral and non-tumoral specimens (data not shown). All PCR conditions were adjusted in order to obtain equivalent optimal amplification efficiency between the different assays. CXCR1, CD3, IL-8, IL-17, Granzyme A, Perforin, and FoxP3 mRNA expression were quantified by the relative quantification of real time-PCR according to Gibson *et al*.(25), using the SYBR Green PCR Kit, and using PBMCs stimulated

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with PMA and ionomycine for 1hr as the calibrator sample. CD3, IL-1β, TNFα, IL-6, IFNγ, IL-4, TGFβ, IL-10, Granzyme B and FasL mRNA expression were quantified by absolute quantification of real time-PCR using the Hybridization Probes PCR Kit from Roche Diagnostics (Meylan, France). The copy number for all target genes and for the HKG was obtained by plotting sample Ct values against the standard curve obtained by analyzing the corresponding "quantitative DNA standard"(26) dilution series using the LightCycler software 4.0, and the magnitude of target gene mRNA expression is calculated as the copy number of the target gene per 10^6 copies of β2μglobulin. All PCR experiments were done in duplicate.

Statistical analysis

Results of the quantification of CD3, IL-8, CXCR-1, IL1β, IL-6, TGFβ, and TNFα expression are presented as the median and interquartile range (IQR) of the ratio of the immune marker gene to HKG mRNA expression. IL-4, IL-10, IL-17, Foxp3, FasL, Perforin, Granzyme A, and Granzyme B are mainly expressed in CD3 mRNA expressing leukocytes. Therefore, to normalize the we used the ratio of mRNA copy number of each marker gene / CD3.

Non-parametric tests were used because of the non-normal distributions of mRNA levels. Overall differences in immune marker expression between the tumoral (T) and autologous non-tumoral (NT) specimens were analyzed by using the paired non-parametric Wilcoxon signed-rank test. For significant variations, gene expression levels between tumoral and non-tumoral specimens were also compared in each phenotypic subgroup (MSI-H and MSS). Differences between MSI-H and MSS phenotypes were evaluated by comparing the T/NT ratio of mRNA expression using the two-sample Wilcoxon rank-sum test. All tests were two-tailed. An adjustment for multiple testing was done using the Bonferroni correction, and p

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values ≤ .003 (0.05/15=.0033) were considered statistically significant. Data were analyzed using Stata Statistical software (StataCorp 2003, Release 8.0, College Station, Texas)

Results

Characterization of patients with CRCs

Patients with the MSI-H phenotype were not significantly different than those with the MSS phenotype in terms of age, diagnosis, therapy, or surveillance procedures (Table1). The rate of tumor relapse within a 36 month follow up was not different depending on MMR status. Tumors with the MSI-H phenotype were right-sided with significantly different histopathology features: they had more lymphocyte infiltration and a greater mucoid component and less local or systemic tumor cell extension.

The MSI-H phenotype correlates with the presence of cytotoxic markers

CD3 mRNA copy number in tumoral (T) tissues was not significantly different than in autologous (NT) tissue fragments [0.18 (0.13-0.28) vs 0. 17 (0.11-0.26)] when all samples were considered (irrespective of MMR status). The CD3 T/NT ratio did not differ between MSI-H and MSS tissues. The expression ratios of the cytotoxic parameters, FasL/CD3, Perf/CD3 and GzB/CD3, were significantly higher in tumor tissues than in autologous NT specimens (Table 2). Adjusting on MMR status, only the perforin T/NT ratio was higher in MSI-H tissues than in MSS tissues. The expression of Gz B/CD3 was significantly higher in tumor (as compared to NT) specimens only if they had an MSS phenotype. The GzA/CD3 ratio did not differ between tumoral and autologous NT specimens.

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MSS phenotype and higher Foxp3 and IL-17 expressions in tumor infiltrates

Overall analysis showed that the expression ratios IL-17/CD3 and Foxp3/CD3 **(Table2),** and IL-8, IL-1β, IL-6, and TGF-β expression **(Table 3),** were significantly higher in tumor tissues than in autologous NT specimens. However, adjusting for MMR status, the higher expression of Foxp3, IL-17, IL-1β, IL-6, and TGFβ was observed only in MSS phenotype tumors **(Tables 2 & 3).** The IL-17 T/NT ratio was higher in MSS tissues than in MSI-H tissues. Moreover, quantification by RQ-PCR of IL-22 expression, another T_H17-secreted cytokine, showed IL-22 is overexpression in MSS tumour tissues compared to MSI-H tumour tissues ($p= 0.042$ for the ratio difference between MSI T/NT and MSS T/NT). The IL-10/CD3, IL4/CD3 ratios **(Table 2),** and CXCR-1 and TNF expression **(Table 3)** did not differ between the tumoral and autologous non-tumoral specimens. Expression of IFNγ was not detected in any specimen.

Immunohistochemistry

IL17 immunoreactive cells were rarely detected in normal tissues in the lamina propria (Figure 1a). However, their number was higher in tumour sections than in autologous normal tissues. IL17 cell infiltration was higher in MSS tumours than in MSI tumours (Figure 1b). Only few number of CD3+ cells in tumours were immunostained with IL17 antibody, too (Figure 1c).

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Discussion

Elucidation of the nature of the intratumoral inflammatory reaction is relevant not only to better understand the pathobiology of CRCs but also to assess the possible influence of host immune response on patient outcome. Our study examined the differences in the pattern of inflammatory and immune response markers expressed in the tumour microenvironment of a series of 45 tumours from patients with sporadic CRC. About one third of the patients had MSI-H colorectal cancer although no one displayed HNPCC syndrome and/or the Bethesda criteria (27). MSI-H phenotype tumors, as assessed by immunohistochemistry, showed more pronounced lymphocytic infiltration than MSS phenotype tumors. The MSI-H phenotype was associated with a higher expression level of cytotoxic cell markers, especially perforin. Conversely, the MMR-proficient (MSS) phenotype was associated with higher expression levels of Foxp3, a marker of regulatory T cells, and of inflammatory (IL-1β, IL-6, IL-8, IL17**) or** suppressive (TGFβ) cytokines, with IL-17 expressed at particularly high levels. No difference was observed between MSI-H and MSS tumors regarding CD4+ Th1 and Th2 cell subset infiltration, as assessed by the mRNA levels of the relevant cytokines (IFNγ, TNFα, and IL-4). Thus, we show for the first time a paradoxical gene expression signature combining markers associated with inhibition of the anti-tumor immune response (TGFβ, Foxp3), and pro-tumoral inflammatory response (IL-17, IL-6) in the microenvironment of sporadic CRCs without microsatellite instability.

Pronounced lymphocytic infiltration is linked with longer survival in human cancers (16;28-31), and, immune suppression clearly enhances the chance of cancer appearance in human and animal models (32). However, the lack of tumour immune surveillance has been

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associated with the context of an inflammatory cytokine milieu (Il-6, Il-1β, TNFα, IL-17)(33;34). Thus, the phenotypic and functional characterization of these lymphocytic infiltrates [i.e. CD8+ T cells (central and effector memory CD8+ T cells) and CD4+ T cells (CD4+ T helper cells of types 1, 2 and 17), and regulatory/suppressive CD4+ CD25+^{high} T cells (Treg)] has been proven to predict prognoses in melanoma, ovarian cancer, and non-Hodgkin's lymphoma (35;36). Together, the data from human studies supports the existence of a cancer-immunosurveillance system that involves Th1-dependent cytotoxic cells and which is locally suppressed by Treg cells and inflammatory products.

In colorectal cancer, the role of tumor-infiltrating inflammatory cells and lymphocytes is still controversial. An increased number of inflammatory cells in tumours may favour tumour progression (21). Increased expression of cytotoxic CD8-positive T cells, and increased apoptosis of tumour cells were demonstrated in small series of MSI-H colorectal cancers by using immunohistochemical methods (17;37;38), by assessment of cytotoxic marker mRNA levels (such as perforin and granzyme B) (39), and by using oligonucleotide microarray analysis and/or quantitative reverse transcriptase-PCR assays (40-42). Different findings support the hypothesis that MSI-H CRCs, that are roughly considered to display a good prognosis (43;44), may lead to the production of genetically altered proteins in the tumour microenvironment. These proteins may function as tumour-specific neoantigens able to elicit potentially effective anti-tumor cytotoxic responses(17). Our results provide additional evidence of the close relationship between pronounced lymphocytic infiltration and the overexpression of cytotoxic markers in MSI-H tumours indicating a better cell-mediated tumor-specific immune response in patients. Although CRCs in general are known as poor immunogenic tumours (45) those with MSI-H phenotype should be considered as immunogenic. However, majority of CRCs are of MSS phenotype. Together, with the low density of CD3 positive lymphocytic infiltrate and the low perforin expression, this phenotype

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could reflect the poor immunogenic capacity to generate a specific cytotoxic CD8+ T cell response. Recently, immunological data, e.g. presence of markers for T_H1 and cytotoxic polarization, the memory T cell type, a high density, and location of immune cells within the tumor samples, were found to be a better predictor of patient survival than the histopathological methods currently used to stage colorectal cancer (46;47). The molecular phenotype of human CRC based on MMR status has not been taken into consideration in these two latter studies. Because MSI-H phenotype, which is usually described in only 15-20% of sporadic CRCs, it is unlikely that all tumours (88 out of 104) presenting with higher density of CD3+ plus CD45+ cells and with longer duration of disease-free survival in related patients, in this series (46;47), might be of MSI-H phenotype. Thus, MSI-H CRC may still be considered as a single prognostic marker in CRC.

We report for the first time the coexistence of higher expression of markers associated with anti-tumor immune response (Foxp3 and TGF-β) and of the two inflammatory cytokines IL-6 and IL-17, in MMR-proficient CRC. The higher expression of Foxp3 in the current study could be the hallmark of either naturally occurring, thymically produced nTreg- or extra-thymically generated Tr1/Th3-cell infiltrates. The extra-thymically T cells are distinct from thymically produced nTreg cells, because they generally don't need contact-dependent mechanisms and may react to soluble cytokines (typically IL-10 or TGF-β (*for review : see (48)*. Although CD25+ CD4+ T cells could be generated, in vitro, from peripheral naïve CD4+ T cells of Foxp3 reporter mice (49), there is no evidence these events could occur in physiological conditions and/or in tumor-induced immune responses *in vivo*. However, these unknowns do not undermine the pluripotential role of TGF-β in the maintenance of Foxp3 expression, regulatory function, and homeostasis in peripheral CD4(+)CD25(+) regulatory Tcells (50), and well established Foxp3-dependent suppressive abilities of extra-thymically generated Tr1/Th3- cells (51). Moreover, the immunosuppressive capability of TGF-β is

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alternatively supported by its ability to prevent the maturation of dendritic cells (DCs) by maintaining a low expression of co-stimulatory molecules (52;53) and by generating DCs that promote Treg-dependent tolerance (54). Alternatively, tumour cells produce soluble factors such as IL-6 that cause a specific dendritic cell subset to secrete bioactive TGFβ, which in turn acts as a co-stimulator of the expansion of Foxp3 expressing T reg cells (55). Thus, in our study, the higher expression of TGF-β, whatever the nature of the TGFβ– secreting cells (e.g. Treg, tumor cells, fibroblasts), could participate to the induction of suppressive mechanisms inducing tumor tolerance in the MSS tumor tissues.

Of potential interest for further functional investigation is analysis of co expression of cytokines e.g. IL-17A-expressing cells, in the context of IL-6- and TGFβ-expression in MSS tumor tissues. Although the differentiation of T_H-17 was described to be determined by exposure to TGF-β and IL-6 (56) (57), we cannot definitively conclude if the changes in cytokine gene expression in MSS tumors are caused by tumor infiltrating lymphocytes or innate immune cells, rather than by tumor infiltrating fibroblasts. Using double staining procedures on IHC, we could show that minority of IL17 immunostained cells were of CD3 phenotype, too. This is consistent with data from literature indicating less than 15% CD4+ conventional T cells are IL-17 (T_H-17) when about 60% of the IL-17A –producing cells are γδT cells, and 25% are NKTlike cells. T_H-17 cells coexpress IL-17 and IL-22 (58). In attempt to further discriminate between the three types of IL-17-secreting T cells, we quantified IL-22 expression in the same set of MSS and MSI-H tissue samples and autologous controls and found higher IL-22 expression levels in MSS tumors than in MSI-H tumor tissues (personal unpublished data not shown). Although indirect, this result supports the hypothesis of a higher infiltration of MSS tumor by T_H-17 cells, potentially linked to the simultaneous higher expression of TGFβ and IL-6 in the microenvironment of MSS tumours. Our results are in agreement with different studies showing that the enhanced tumour growth

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elicited by IL-17 was associated both with its pro-angiogenic effect (59) and with the increased expression of IL-6 at the tumour site (60). Our data are also consistent with a previous report conducted in atumour murin model. It showed that IL-17 may exert protumor or antitumor effects, depending on the immunogenicity of the tumor and the presence of specific cytolytic T lymphocytes (61). Although further investigations are needed to clarify the cellular sources of TGFβ, IL-6, IL-17 (35;62) we think it is worth considering that the poorly immunogenic MSS tumour might generate regulatory T cells instead of anti-tumor effector cytotoxic cells, leading to exacerbate, and not to control, the disease. We would suggest the coexistence of higher Foxp3, IL-6, TGF-β and IL17 expression represents potential biomarker for the future development of a new prognostic "test set" for sporadic colon cancer.

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TABLE 1: Characteristics of the study population

Parameters	All cases		MSS		MSI-H		P value*
	(n=45)		(n=31)		(n=14)		
	n	(%)	n	(%)	N	(%)	
Gender (F)	21	(47)	11	(35)	10	(71)	.05
Mean age at surgery (± sd)	69.7 ± 1.5		68.8 ± 1.8		71.8 ± 2.8		.40
Right sided tumor	19	(42)	9	(29)	10	(71)	.01
No or poor differentiation	27	(60)	20	(70)	7	(50)	0.21
Mucous component	4	(1)	0	(0)	4	(40)	.01
Lymphocyte infiltration†	14	(31)	1	(3)	13	(93)	.0001
Tumor staging							
pT 1-2	4	(9)	2	(6)	2	(14)	
pT 3	27	(60)	16	(52)	11	(79)	
pT 4	14	(31)	13	(42)	1	(7)	.05
N0	26	(58)	15	(48)	11	(79)	
N1-2	19	(42)	16	(52)	3	(21)	.10
Synchronous metastasis	22	(49)	19	(61)	3	(21)	.02
Vascular emboli	25	(56)	20	(65)	5	(36)	.03
Perinervous infiltration	7	(16)	5	(16)	2	(14)	1
Relapse within 36 months of follow up	10	(22)	8	(26)	2	(14)	.47 ‡

† Assessed by histopathology evaluation (yes=+ to ++; no=0 to +/-).
* p value of Fisher exact test or Mann-Whitney test as appropriate.
‡ p value of Log-rank test for equality of survivor functions.

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TABLE 2: Gene expression levels/CD3 in colon cancers compared to normal mucosa

Target gene expression	n	Tissues		*P value**	T/NT ratio
		Non-tumoral	Tumoral		
Fas Ligand	(45)	1390 (463-2055)	2294 (964-3842)	***.000***	
MSS	(31)	1500 (274-2040)	1731 (708-3376)	*.01*	1.7
MSI	(14)	1275 (750-2154)	3305 (2031-7821)	*.02*	2.6
P value†					*.68*
Perforin	(45)	13.2 (8.1-17.9)	19.3 (13.2-41.5)	***0.002***	
MSS	(31)	13.5 (8.8-20.9)	17.1 (11.3-35.1)	*.07*	1.2
MSI	(14)	8.9 (5.4-18.5)	38.5 (18.5-69.0)	*.009*	3.2
P value†					***.003***
Granzyme A	(45)	1.6 (1.0-2.6)	1.6 (1.0-2.0)	*.72*	
MSS	(31)	1.6 (1.2-2.5)	1.5 (.9-2.0)		
MSI	(14)	1.6 (.9-2.6)	2.1 (1.6-3.5)		
P value†					
Granzyme B	(45)	5555 (2992-11501)	19065 (14440-30586)	***.0000***	
MSS	(31)	5545 (2586-7640)	16289 (9396-23507)	***.0000***	3.4
MSI	(14)	7915 (4167-14431)	24758 (17223-61150)	*.006*	4.1

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P value†					*.71*
IL-4	(45)	99 (48-421)	238 (120-450)	*.06*	
MSS	(31)	81 (46-421)	238 (120-450)		
MSI	(14)	124 (50-515)	203 (91-471)		
P value†					
IL-10	(45)	227 (88-529)	284 (199-527)	*0.06*	
MSS	(31)	250 (76-646)	284 (203-509)		1.7
MSI	(14)	154 (106-529)	317 (143-654)		1.2
P value†					*.68*
IL-17	(45)	.6 (.2-1.8)	4.5 (1.8-11.5)	***.0000***	
MSS	(31)	.5 (.2-1.8)	7.2 (2.3-18.8)	***.0000***	13.7
MSI	(14)	1.4 (.6-2.0)	2.4 (.4-4.4)	*.30*	1.4
P value†					***.002***
Foxp3	(45)	2.7 (1.7-4.0)	15.6 (11.2-20.1)	***.0000***	
MSS	(31)	2.6 (1.7-3.9)	16.0 (13.7-21.0)	***.0000***	6.8
MSI	(14)	3.1 (1.7-6.2)	10.5 (3.9-19.7)	*.07*	3.2
P value†					*.04*

Results are expressed as median (first-third quartiles) of the ratio of target gene to CD3 mRNA copy numbers.
* P value of the paired non-parametric comparisons between tumoral and non tumoral tissues (Wilcoxon signed-rank test).
† P value of the non-parametric comparisons between MSS and MSI-H phenotypes.
After using the Bonferroni correction for multiple testing, P ≤ .003 was considered statistically significant. The P values are in bold.

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TABLE 3: Gene expression levels in MSI-H and MSS colon cancers compared to normal mucosa

Target gene expression	n	Tissues		*P value**	T/NT ratio
		Non tumoral	Tumoral		Non tumoral
IL8	(45)	.3 (.1-.7)	7.5 (3.4-12.2)	***.0000***	
MSS	(31)	.2 (.1-.7)	7.5 (3.4-11.0)	***.0000***	35.5
MSI	(14)	.4 (.1-.9)	9.8 (3.0-16.5)	***.001***	26.0
P value†					*.79*
CXCR1	(45)	.2 (.1-.4)	.6 (.3-1.3)	*.004*	
MSS	(31)	.2 (.1-.4)	.5 (.2-.7)		
MSI	(14)	.2 (.001-.8)	.9 (.4-2.9)		
P value†					
IL-1β	(45)	336 (104-1047)	2143 (686-4729)	***.0000***	
MSS	(31)	330 (73 – 622)	1852 (489-3392)	***.0000***	4.7
MSI	(14)	388 (172-1290)	3844 (686-5756)	*.01*	4.5
P value†					*.79*
IL-6	(45)	1014 (297-4837)	12281 (5942-23507)	***.003***	
MSS	(31)	904 (247-4815)	12824 (3597-28160)	***.002***	19.6
MSI	(14)	1764 (726-11418)	11357 (7165-17411)	*.36*	3.6
P value†					*.08*

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TGFβ	(45)	1878 (975-2992)	4488 (2191-6061)	***.0000***	
MSS	(31)	1755 (962-3030)	3941 (1953-5935)	***.0000***	2.2
MSI	(14)	2007 (1054-2728)	5264 (2818-9656)	*.004*	2.5
P value†					*.71*
TNFα	(45)	133 (75-269)	216 (153-390)	*.22*	
MSS	(31)	104 (65-234)	199 (139-381)		
MSI	(14)	192 (130-1359)	222 (174-492)		
P value†					

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Figure 1

Title: IL17 immunoreactive cells in colonic tissues

Legends: IL17 producing cells are mainly located in the lamina propria in the normal colonic mucosa (a, left with higher magnification x 60 in the bottom) as compared to the control (in right) with the main antibody omitted during the immunohistochemistry reaction. IL17 immunostained cells infiltrated tumours with MSS phenotype (b, left) in a proportion higher than in those with MSI phenotype (b, right). CD3 immunostaining in a colon tumour section (c, left) as compared with double staining reaction using CD3 and IL17 (brown and red, respectively) in the same colon cancer (c, right with MagX20 and higher magnification x60 of some double stained cells in the bottom); Mag: magnification on optic microscopy.