Differences in expression profiling and biomarkers between histological colorectal carcinomas subsets from the serrated pathway.

Running title: Signature and biomarkers in serrated carcinomas

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The authors declare no conflict of interests

• **A word count (from beginning of Introduction to end of Discussion)**

Abstract

Background. Colorectal carcinomas (CRC) from the serrated route like serrated adenocarcinoma and CC showing molecular features of microsatellite instability (hmMSI-H) share common features (preference for female genre, right side location, mucinous histology and altered CpG methylation patterns) but dramatically differ in terms of prognosis, development of immune response and treatment options. Despite this fact, to date no expression profiling comparison was carried out to find out which functions and genes may be responsible for such differences. Methods. The molecular signatures of SAC and hmMSI-H were obtained with transcriptomic arrays and qPCR and immunohistochemistry (IHC) were used to validate differentially expressed genes at mRNA and protein level. Results. An over-representation of innate immunity functions (granulomonocytic recruitment, chemokine production, TLR signaling, antigen processing and presentation) were obtained from this comparison and ICAM1 was more expressed in hmMSI-H whereas two genes (CRCP and CXCL14) were more expressed in SAC. These array results were subsequently validated by qPCR and CXCL14 and ICAM1 by IHC. Conclusions. Our findings point out specific functions and genes which provide a better understanding about the role of immune response in serrated pathological route and may be of help for identifying particular molecular targets, especially in SAC which lacks molecular targeted therapy.

Introduction

The so-called adenoma-carcinoma sequence is typically characterized by chromosomal instability and microsatellite stability (MSS) and by ending up in the development of conventional carcinoma (CC) [Vogelstein]. Less is known about the CRCs being the end-point of the serrated pathway although high-level of microsatellite instability (MSI-H), BRAF mutation and CpG island methylation phenotype (CIMP) seems to be driven forces in this carcinogenic process [Makinen, 26].]. Based on common features as right sided location and the identification of remnants of serrated polyps (SP) adjacent to the tumor, it was assumed that serrated adenocarcinoma (SAC) and CRC showing histological and molecular features of MSI-H [10] (hMSI-H) are both end-points of the serrated pathway [108]. Strikingly, SAC and hMSI-H differ in terms of prognosis, and treatment options. SAC has been recognized in the latest WHO classification of tumors of the digestive system as a new subtype of colorectal cancer (CRC)[1], accounting for 7.5 to 8.7% of all CRCs[2,3]. Criteria for its histologic diagnosis have been proposed [2] and validated in a series of 81 cases [3] and it has been shown to have a worse prognosis than conventional carcinoma (CC) [3] displaying a higher frequency of adverse histological features at the invasive front including a weak peritumoral lymphocyte response [4]. On the contrary, hMSI-H is characterized by the occurrence of Crohn-like reaction and peri- and intra-tumoral infiltrates [10]. Moreover, it has been recently demonstrated that, based on higher expression of PD-L1 and BRAF mutation, hMSI-H patients are candidates for immune checkpoint and tyrosine kinase inhibitors, respectively [95,96]. In contrast, the frequency of KRAS mutation in SAC is even high than in CC [12, 13] and SAC display mostly MSS thus making them refractory to anti-EGFR monoclonal antibodies and immune checkpoint inhibitors. For these reasons, the aim of this work is, by using molecular profiling, to ascertain which molecular features

are responsible for such differences in immune response between SAC and hMSI-H with a view to identify useful diagnostic biomarkers.

Material and Methods

Patients and tumor samples

The clinico-pathological features of the patients have been previously reported.[3,4] and approval for the study was granted by the Local Ethical Board. SACs were diagnosed on the basis of criteria proposed by Mäkinen et al.[2] and hMSI-Hs according to prior established criteria (mucinous, signet-ring cell, and medullary carcinoma, tumor infiltrating and peritumoral lymphocytes, "Crohn-like" inflammatory response, poor differentiation, tumor heterogeneity, and "pushing" tumor border).[10] Frozen samples of 11 SACs and four hMSI-H were retrieved from the Santa Lucia University Hospital, Cartagena, Spain and used for the gene expression microarray study. Validation by qPCR was performed upon frozen specimens of 12 SAC and nine hMSI-H and, in addition, adjacent normal mucosa was also analyzed from seven SACs and six hMSI-H. Paraffin blocks of 10 SAC and 15 matched hMSI-H, included in previous works, [8,13] were used for immunohistochemistry (IHC) validation. Clinico-pathological features of the study cases are shown in Table 1. The study was approved by the Hospital Ethics Committee and was carried out in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. Written informed consent was obtained from all patients.

RNA extraction

A volume of approximately 10mm^3 was extracted from each frozen tissue using the disposable sterile biopsy punch Acupunch 2mm (AcudermInc, Lauderdale, FL, USA). RNA was extracted following the manufacturer´s instructions (Qiagen, Hilden, Germany). Briefly, tissue was disrupted and homogenized in 700µl of Qiazol (Qiagen ref:1023537) using a Tissueruptor by Qiagen for 20 seconds. The homogenate was incubated at room temperature for 5 minutes. After adding 140µl of chloroform and centrifuging at 12,000xg for 15 minutes at 4ºC, 350µl of the aqueous phase was subjected to automatic total RNA extraction using the Qiacube equipment and the miRNeasy Mini Kit (ref:217004), both provided by Qiagen.

RNA labelling and microarray hybridisation

Total RNA was quantified by spectrometry (NanoDrop ND1000, NanoDrop Technologies, Wilminton, Delaware USA) and fragment size distribution was analysed by RNA 6000 Pico Bioanalyzer assay (Agilent Technologies, Palo Alto, California USA). RNA 150ng was concentrated in a SpeedVac to a working dilution and used to produce Cyanine 3-CTP-labeled cRNA using the Low Input Quick Amp Labelling Kit, One-Color (Agilent p/n 5190-2305) according to the 'One-Color Microarray-Based Gene Expression Analysis' protocol Version 6.0 (Agilent p/n G4140-90040). This method uses T7 RNA polymerase which simultaneously amplifies target material and incorporates cyanine 3- labelled CTP. A 2000ng cRNA product was hybridized with Whole Human Genome Oligo Microarray Kit (Agilent p/n G2519F-014850) containing 41,000+ unique human genes and transcripts. Arrays were scanned in an Agilent

Microarray Scanner (Agilent G2565BA) according to the manufacturer's protocol and data extracted using Agilent Feature Extraction Software 10.7.1 following the Agilent grid template 014850_D_F_20100430 protocol GE1_107_Sep09 and the QC Metric Set GE1_QCMT_Sep09.

Microarray data analysis

Agilent raw data were pre-processed using Agilent background correction and quantile normalisation was applied to obtain homogeneous scales in all samples. Differential expression analysis was performed on normalized data using the Limma (Linear Models for Microarray Data) package by Bioconductor (www.bioconductor.org/packages/2.3/bioc/html/limma.html) and comparison was made between SAC (n=11) and hMSI-H (n=4). The p-values were corrected by multiple testing using the Benjamini and Hochberg method[90] to give adjusted p-values. In order to further identify cellular function differences between SAC and hMSI-H, gene expression data were analysed by Gene Set Enrichment Analysis (GSA) using the FatiScan tool of the Babelomics suite (www.babelomics.org).[91] We used different functional annotation databases, namely the pathways from the KEGG database (www.genome.jp/kegg) and the Biological Process, Molecular Function and Cellular Component from Gene Ontology (GO) (www.geneontology.org). Paintomic representations on significant KEGG pathways were used to portray those proteins whose gene expression was higher in SAC (blue boxes) or higher in hMSI-H cases (red boxes), the intensity of the colour indicating the strength of this difference [92]. Differentially expressed GO biological process were represented as scatterplot and TagPlots using REVIGO online package [93].

Quantitative PCR

The retrotranscriptase reaction was performed from a total of 1 μg of DNAseI-treated RNA using the DyNAmo cDNA synthesis Kit (ref:F470L) provided by Thermo Scientific (Rockford, IL). Five microlitres of 1:5 diluted cDNA was added to the qPCR reaction containing 12.5μl 2X QuantiTect SYBR Green PCR Kit (ref:204145) by Qiagen and 300nM of each primer in a total volume of 25μl. qPCR was performed on a 7500F real time PCR system by Applied Biosystems (Foster City, CA, USA) according to the instruction manual and following the standard protocol: 50ºC 2min, 95ºC 10min, 40 cycles of 95ºC 15sec, 60ºC 1min and a melt curve stage consisting in 95ºC 15sec, 60ºC min, 95ºC 30sec and 60ºC 30sec. The relative quantitation was done by the 2-ΔCt method using β-actin as housekeeping gene. Primers were designed by using primer3 software and sequences and fragment size are described in Table 2.

Immunohistochemistry

The validation subset consisted of 40 SC and 20 hMSI-H cases matched for gender, age, location. Blocks from each case were selected based on larger tumor invasive front areas and all stainings were performed on whole tissue sections using the Benchmark Ultra Ventana and the Optiview DAB IHC V5 kit. Details on antibody (purveyor, reference, type, (clone); antigen retrieval conditions (buffer, temperature, time); incubation (temperature, time) and dilution are as follows: CRCP. Sigma Aldrich, HPA007216, polyclonal; CC1, basic, 95ºC, 56min; and room temperature (RT), overnight (ON) and 1:350. CXCL14: Abcam, ab3662, polyclonal; C1, basic, 95ºC,

56min; RT, ON and 1:100. ICAM1: Cell Signalling Tech, #4915, polyclonal; CC2, acid, 95ºC, 48min; RT, ON and 1:300. Endogenous peroxidase activity was blocked using 0.5% H_2O_2 for 5 minutes. For visualization of the antigen, the sections were immersed in 3,3'-diaminobenzidine (DAB) and counterstained with Harris´ haematoxylin for 5 minutes. As controls, normal liver was used for CRCP, brain for CXCL14 and kindney for ICAM1 as suggested by the antibody purveyors. These markers were evaluated at the tumor invasive front by considering a staining intensity score (1=none or weak staining, 2=moderate, 3=strong) in a given area and a stained area score (A< one-third, B=between one- and two-thirds, C> two-thirds). For statistical analysis, both intensity and distribution were considered and those immunoscores being 1B or less were assumed as negative whereas those being 2B or higher were considered positive.

Statistical analysis

Statistical analysis was performed using SPSS (Version 15.0, Chicago, IL) package. For checking the relationship between mRNA expression by qPCR and histological diagnoses the Mann-Whitney's *U* test was used. Statistical significance in the immunohistochemistry study was assessed using Pearson χ^2 or Fisher´s exact test when indicated.

Results

Differentially expressed functions

Bioinformatic analysis revealed a considerable number of KEGG pathways and Gene Ontology terms differentially expressed in SAC vs. hMSI-H (levels from 3 to 19): 56 KEGG pathways; 529 GO biological processes (BP); 56 GO cellular component (CC) and 101 GO molecular functions (MF). As shown in supplementary material S1, apart from differentially represented KEGG pathways dealing with neurodegenerative diseases (hsa05014, hsa05012), autoimmune diseases (has04940, hsa05320) and protein and aminoacid metabolism (hsa00270, hsa00280, hsa03050, hsa00310, hsa00380) it is remarkable the over-representation of immune response pathways (intestinal IgA production (hsa04672, leukocyte transendothelial migration (hsa04670), NOD- (hsa04621) and Toll-like receptor signalling (hsa04620), Adipocytokine signalling (hsa0920), complement cascade (hsa04610), antigen procession and presentation (hsa04612), primary immunodeficiency (hsa05340), Fc epsilon RI signaling pathway (hsa04664), B cell receptor signalling pathway (hsa04662), Fc gamma R-mediated phagocytosis (hsa04666), allograft rejection (hsa05330), Graft-versus-host disease (hsa05332). Likewise, differentially expressed GO biological processes were related to immune response, such as antigen processing and presentation, humoral immunity and cytokine (Fig. 1). More precisely, supplementary material S1 points out molecular functions associated with neuropeptide binding, MHC and chemokine activities, G-coupled receptors and GTPases, NADH dehydrogenase activity and actin binding. Interestingly, when KEGG cytokine-cytokine receptor interaction panel was represented with Paintomics, it was observed that the interactions of some cytokines (especially chemokines from the C-X-C family) were typical from

hMSI-H (CXCL10, CXCL11, IL8, OSM, IL-1B, IFNG and CSF2) whereas in SAC almost exclusively involved CXCL14. Other cytokine families such as TGF-β and IL17 were less differentially implicated in SAC and hmMSI-H (Supplementary material S2A). KEGG colorectal cancer panel also showed a dissimilar representation of pathways in the comparison of these tumour types whereas the antigen processing and presentation panel showed an over-representation in hmMSI-H of molecules particularly involved in MHC class I pathway (Supplementary material S2B and S2C, respectively).

Differentially expressed genes

The analysis of microarray data identified 1,144 differentially expressed genes, 533 of which were more expressed in SAC than in hmMSI-H and 611 more expressed in hmMSI-H. A list of the 34 genes; 17 more expressed in SAC and 17 in hmMSI-H is provided as Supplemental material S1. In general terms, genes overexpressed in SAC play roles in GTPases signalling and in apoptosis / cell cycle control (GPR56, CRCP, PLEKHQ3, TUFT1, FANK1, CDK20, CDK6) whereas those over-represented in hmMSI-H were more involved in immune response (LILRA3, CSF3R, CCL3L3, ICAM1, FCGR2B, LILRB3, amongst others). Based on the extent of differential expression grade, the importance of the biological functions, the design of suitable primers and the availability of antibodies we decided to validate *CXCL14, CRCP* and *ICAM1* by qPCR and IHC. According to microarray results, CRCP and CXCL14 were overexpressed in SAC whereas ICAM1 was overexpressed in hmMSI-H.

Validation by qPCR

Gene expression of *CRCP, CXCL14* and *ICAM1* was assessed by quantitative PCR in a series incorporating more hmMSI-H cases and one more SAC. In order to quantify the presence of such genes in normal tissue, healthy mucosa adjacent to tumor was also analyzed. As shown in Figure 2, when comparing tumor and normal mucosa, CXCL14 was more expressed in normal than in tumor tissue (7.35 ± 2.2 vs. 3.63 ± 1.2; p=0.002); especially when hmMSI-H tumors were compared to their corresponding adjacent mucosa (9.64 ± 4.4 vs. 1.26 ± 0.7; p=0.003). In contrast, *ICAM1* was more expressed in tumoral than in normal tissue (0.03 ± 0.009 vs. 0.009 ± 0.003; p=0.019), this difference being more relevant in hmMSI-H cases where tumor tissues expressed 10 times more *ICAM1* than neighboring healthy mucosa (0.07 ± 0.03 vs. 0.007 ± 0.003; p=0.003). qPCR assays on cancer specimens validated microarray; *CRCP* and *CXCL14* were more expressed in SAC than in hmMSI-H (0.22 ± 0.07 vs. 0.04 ± 0.02; p=0.001 and 4.9 ± 1.7 vs. 1.2 ± 0.7; p=0.003, respectively) whereas ICAM1 was more expressed in hmMSI-H than SAC (0.07 ± 0.03 vs. 0.01 ± 0.003; p=0.003).

Validation by immunohistochemistry

In order to investigate whether differential expressed genes would have an impact on protein expression, immunohistochemistry was performed in an extended series. CRCP showed a membranous and cytoplasmic staining in normal colorectal epithelial cells with no expression on lamina propria cells; CXCL14 was also cytoplasmic and was found in neuroendocrine cells from the epithelial crypts in cells from the lamina propria whereas ICAM1 expression was absent in colon glands and present in different stromal

cells including endothelial cells (Fig 3). CRCP and CXCL14 were also expressed in tumor cells whereas ICAM1 was expressed in stromal cells and, stainings were evaluated in the corresponding type of cell, accordingly. Results are shown in Table 4 and representative staining images in Figure 4. CRCP staining was not significantly associated with tumor subtype as positive expression was not more frequently observed in SAC compared to hmMSI-H (45% vs. 50%; p=0.463). Nevertheless, and in agreement with the array results, CXCL14 was more expressed in SAC than in hmMSI-H (14/40 (35%) vs. 2/20 (20%); p=0.0353) and ICAM1 more expressed in hmMSI-H than in SAC (15/20 (75%) vs. 18/40 (45%); p=0.0194.

Discussion

Despite recent different molecular classifications for colorectal cancer have been proposed with a grade of overlap [Guinney, Rodriguez-Salas], CRCs are currently diagnosed based their histology [1] and just a few biomarkers are currently used to determine the most suitable treatment. It is for this reason important to correlate molecular profiling with histological features, this issue being especially critical in the serrated pathway for colorectal carcinogenesis since it is not as clearly discerned as the conventional adenoma-carcinoma. Furthermore, the immune surveillance awake against tumour is now considered as a breakthrough in cancer treatment and in the serrated pathological pathway two CRC subtypes can be found with typical weak (SAC) or abundant (hMSI-H) immune responses. Therefore, as no previous studies have compared the molecular features of serrated pathway CRCs, we aimed to characterize the biology of these tumours in order to identify which steps in the complex immune response against the tumor could need to be targeted for giving SAC a specific

histology-based treatment. With no prior selections of functions our results demonstrate that the immune features are what basically make SAC and hMSI-H different at the molecular level. More specifically, activities dealing with innate immunity, antigen processing via MHC class I, chemokines from the C-X-C family and transendothelial leukocyte migration are more characteristic of hmMSI-H. In fact, the main immune response against cancer involved tumour antigen presentation by class I MHC and subsequent CD8 T cell activation. Interestingly, amongst most significantly over-expressed genes in hmMSI-H (Table 3) there is an important representation of those coding for proteins involved in carbohydrate ligand binding implicated in immune response, such as SIGLEC5 which codes for a cell surface lectin with sialic acid recognition sites for the first Ig V set domain [Barb]. In addition, CLEC4D and CLEC4A also, over-expressed in hmMSI-H, encode for members of the C-type lectin/C-type lectin-like domain (CTL/CTLD) superfamily which have diverse functions, such as cell adhesion, cell-cell signalling, binding to carbohydrate endogenous and pathogenic ligands, and playing roles in inflammation and immune response acting as pattern-recognition and antigen-uptake receptors and also signalling cytokine production [Meyer-Wentrup]. A very interesting molecule in immune response activated by pathogen carbohydrates and cytokines, such as TNF-α and IL-1, is the intercellular cell adhesion molecule-1 (ICAM1 or CD54), a cell surface glycoprotein that belongs to the immunoglobulin superfamily (IgSF) of adhesion molecules [101]. ICAM1 functions as a co-stimulator on antigen presenting cells, binding to its receptor LFA-1 (leukocyte function associated antigen-1) on the surface of T cells during antigen presentation [102]. Apical localization of ICAM1 on endothelial cells (or basolateral localization on epithelial cells) is a prerequisite for leukocyte trafficking through the endothelial (or epithelial) barrier, thus mediating pathogen invasion as well as host

defense, a pattern also observed in tumors [101]. Given that in hmMSI-H, the over-expressed gene ICAM1 is implicated in functions characteristic of this tumor type such as antigen processing and presentation, cytokine production and leukocyte transendothelial migration, we decided to validate this gene using qPCR and IHC. The results confirm at the mRNA and protein levels that ICAM1 is over-expressed in hmMSI-H compared to SAC and when comparing tumour versus normal adjacent mucosa, ICAM1 was found to be more expressed in tumor than in normal tissue. In agreement with our findings, Astarci et al reported, using tumor samples from colon cancer patients, that non-transformed normal cells (well-differentiatied) showed no immunohistochemical expression of ICAM1, but the poorly differentiated tumor cells showed higher expression [Astarci] Moreover, the incidence of lymph node or liver metastasis was significantly lower in patients with ICAM1-positive tumors than in those with ICAM1-negative tumors, the presence of tumour infiltrating lymphocytes being more frequently observed in the ICAM1-positive tumors than in the ICAM1-negative tumors. The prognosis of the patients with ICAM1-negative tumors was significantly poorer than that of those with ICAM-1-positive tumors [Maeda]. All these findings are concordant with ours as hmMSI-H cancer cells are less differentiated than typical gland-forming SAC cells and develop higher immune response at the tumor microenvironment. Other reports have related immunohistochemical ICAM1 expression with better prognosis as it was inversely associated with M2 macrophage infiltration and with the metastasis index in human colon tumors and that loss of ICAM1 accelerated liver metastasis of colon carcinoma cells. [Yang]. More specifically, ICAM1 downregulation has been associated with immunosuppressant signals; Michielsen et al. observed that pre-treatment of monocyte derived dendritic cells with tumour conditioned media obtained from colorectal tumour explant culture inhibited the

up-regulation of ICAM1 in response to LPS, enhancing IL-10 while reducing IL-12p70 secretion [Michielsen]. Likewise, it has been demonstrated that mature miR-222 and -339 suppress ICAM1 expression on tumor cells, thereby down-regulating the susceptibility of tumor cells to cytotoxic T lymphocytes (CTL)-mediated cytolysis [Ueda] and some other authors showed that the enhanced expression of the costimulatory molecules CD40, CD48 and ICAM1 on target cells results in an increased state of stimulation of CD8+ T cells, and consequent increased lysis of target cells, this effect being mediated mainly through ICAM1 [Slavin-Chiorini]. By studying colorectal carcinoma tissue from 96 patients, Maurer et al. concluded that the over-expression of ICAM-I might prevent cell-cell disruption and, hence, tumor dissemination, and this expression might favor host anti-tumor defense by trafficking of lymphocytes [Maurer].Taking together these reports it could be inferred that ICAM1 is an important molecule favoring the leukocyte migration to the hmMSI-H microenvironment and being able to contribute to an efficient tumor antigen presentation and subsequent activation of CTL in this CRC subtype.

Previous works comparing SAC and CC molecular signatures highlighted that anti-apoptosis, neural differentiation, cytoskeleton, GTPases, calcium signaling, response to hypoxia, tyrosine kinase receptor (TKR)-ERK pathways and the Wnt/β-catenin pathway are characteristic activities of SAC [Laiho, Conesa-Zamora IJC, García-Solano IJC, Conesa-Zamora Clin Epigen]. Accordingly, amongst the most expressed genes in SAC compared to hmMSI-H, some of these functions are also present: apoptosis (*FANK1*) [Wang], neural markers (*GTF2IRD2, CRCP, TUFT1*), cytoskeleton (*MTMR8)*, GTPases (*GPR56, PLEKHG3, CRCP*), calcium binding (*KCNMB3, CRCP*), response to hypoxia (*TUFT1*), TKR-ERK signaling (*IGFBP2, CDK6, CDK20*) and Wnt/β-catenin pathway

(*AXIN2, FRZB*). Given its role as part of a receptor complex for a small neuropeptide, its participation in the regulation of GTPase function, in calcium signaling and in the granulocytic differentiation, *CRCP* was chosen to validate the microarray result as upregulated in SAC compared to hmMSI-H. According to our results, *CRCP* might play some part in the neoplastic process as it is more expressed in tumor than in normal adjacent mucosa. In agreement with the array *CRCP* was over-expressed in SAC as assessed by qPCR. However, we could not validate this finding at the protein level. Possible explanation for this fact could be that, as immunohistochemistry does not give information on protein functionality or isoform type, nor whether the protein is accumulated but not recently expressed nor which of one of the five protein-coding transcripts of *CRCP* is being detected. CRCP is part of a membrane receptor complex (CGRP) required for signal transduction at calcitonin gene-related peptide inducing, by interacting with GTPases, the activation of protein kinases A and C by increasing cAMP and calcium level in the cytosol, respectively and subsequently, activating many potential downstream effectors in cells [Evans]. Previous works have reported a role of CRCP in enhancing the formation of granulomonocytic, but not erythroid or mixed, colonies by purified human CD34(+) cells [Harzenetter]. This role in granulopoiesis might have some consequences in the differentiation of the granulocytic population in the tumour microenvironment, thus favoring a tolererogenic immune state. This hypothesis deserves future studies like evaluating the effect on proliferation and invasion of the reported stable peptide antagonists to CGRP in the tumour *milieu* [Miranda].

Our array results also pointed out CXCL14 (also known as BRAK) as another interesting molecule with implications in the immune regulation. CXCL14 is a

chemokine from the C-X-C family which, in contrast to other members of this group, is upregulated in SAC compared to hmMSI-H. With the aim of finding some clues of whether CXCL14 could have a role in the shift from reactive to tolerogenic immune response, we decided to validated it by qPCR and IHC, thus observing an agreement with array results using these two techniques. Previous works have associated stromal CXCL14, as we evaluated in CRC, with shorter survival in ovarian and breast cancer [Zhao, Sjöberg]. Of note, this marker was more expressed in basal-like breast carcinoma cases which shares characteristics with SAC such as bad prognosis and Fascin1 over-expression [Rodríguez-Pinilla, Conesa-Zamora IJC]. Whereas the stromal expression of CXCL14 seemed to be associated with worse prognosis, some studies, including the transfection of the gene in cancer cell culture, indicated a tumor suppressor role of CXCL14 [Lin]. In agreement with our results, Knight et al. observed, using RNAseq, that CXCL14 expression in sigmoid and rectum is mainly stromal and not epithelial [Knight] and Lin et al. reported that mRNA and protein CXCL14 expression were markedly reduced in colorectal carcinoma compared to normal tissues [Lin] as it was previously observed for other cancer types [Cao]. Interestingly, CXCL14 is a potent chemoattractant for neutrophils, and weaker for dendritic cells, but inactive to monocytes, NK cells, and T and B lymphocytes. [Cao]. This cytokine is implicated in the homeostasis of monocyte-derived macrophages rather than in inflammation and it was reported that a CXCL14-driven recruiting of immature dendritic cells and regulatory T cell (Tregs) after a stroke [Lee]. Using recombinant CXCL14, Shellenberger et al. showed *in vitro*, that this chemokine bound immature dendritic cells with high affinity and blocked endothelial cell chemotaxis [Shellenberger] thus, possibly making difficult the transendothelial migration of immune cells to the tumor environment as opposite of ICAM1. In turn, Meuter et al, using a murine model,

demonstrated that CXCL14 was dispensable for the homeostatic recruitment of antigen-presenting cells toward the periphery and for Langerhans cells functionality [Meuter]. All these evidences might play an important role in SAC by recruiting immunotolerogenic cells, such as Treg and immature dendritic cells, and not those cells involved in fighting against the tumor, which are typically NK and T cells, thus enabling the immune escape characteristic of this tumor type. Moreover, it has been demonstrated that hypoxia inducible factor-1α (HIF-1α) drives CXCL14 expression via directly binding to the CXCL14 promoter [Lee] being this evidence particularly relevant as upregulation of HIF-1α and response to hypoxia are characteristic of SAC compared to conventional colorectal carcinoma [Laiho, Tuomisto, Conesa-Zamora].

Although it is not yet clear the role of ICAM1, CRCP and CXCL14 in cancer and conflicting findings have been reported, it seems that *in vivo* studies are needed to unveil their contribution in the recruitment of granulocytic and monocytic populations in the tumor microenvironment and how this impact the immune response against colorectal cancer. In conclusion, our study suggests that the dramatic differences of SAC and hmMSI-H in terms of immune response against the tumor might be due to distinct functions associated with the innate immunity such as endothelial activation, granulomonocytic recruitment, antigen presentation via MHC class I and chemokine production and this information could be of help for the treatment of SAC, which lacks options of molecular targeted therapy.

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Tables

Table 1. Demographic and pathological features of the study cases.

| | microarray training set | | | qPCR validation set | | | IHC validation set | | |
| | SAC | hmMSI-H | | SAC | hmMSI-H | | SAC | hmMSI-H | |
	n=11 (%)	n=4 (%)	p-value	n=12 (%)	n=9 (%)	p-value	n=40 (%)	n=20 (%)	p-value
Gender									
Female	5 (45.5)	4 (100)		10 (43.5)	7 (77.8)		7 (70)	8 (53.3)	
Male	6 (54.5)	0 (0)	0.029	13 (56.5)	2 (22.2)	0.080	3 (30)	7 (46.7)	0.405
Age (SD)	65.1[22.4]	61 [22.6]	0.759	68.5 [15.99]	66.4 [15.22]	0.942	71.5 [8.8]	66.3 [14.0]	0.449
Localization									
Proximal	9 (81.8)	4 (100)		16 (69.6)	9 (100)		8 (80)	14 (93.3)	
Distal/rectum	2 (18.2)	0 (0)	0.360	7(30.4)	0 (0)	0.061	2 (20)	1 (6.7)	0.315
Dukes´stage									
A	3 (27.3)	0 (0)		4 (17.4)	0 (0)		0 (0)	0 (0)	
B	1 (9.1)	2 (50)		4 (17.4)	4 (44.4)		2 (20)	6 (40)	
C	7 (63.6)	2 (50)	0.162	15 (65.2)	5 (55.6)	0.169	8 (80)	9 (60)	0.294
WHO grade									
High	1 (9.1)	0 (0)		12 (52.2)	0 (0)		4 (40)	1 (6.7)	
Low	10 (90.9)	4 (100)	0.533	11 (48.8)	9 (100)	0.006	6 (60)	14 (93.3)	0.041
Type									
Non-mucinous	7 (63.6)	2 (50)		13 (56.5)	6 (66.7)		8 (80)	12 (80)	
Mucinous	4 (36.4)	2 (50)	0.634	10 (43.5)	3 (33.3)	0.597	2 (20)	3 (20)	1.000

SAC: Serrated adenocarcinoma; hmMSI-H: Colorectal carcinoma showing molecular and histological features of microsatellite instability; qPCR: quantitative polymerase chain reaction. IHC: Immunohistochemistry. SD: Standard deviation. WHO: World Health Organisation

Table 2. Sequences and amplicon sizes for the primers used in this study

Gene	Primer sequence (5´-3´)	fragment size (bp)
CRCP	Fw: GCCACAAGTTGACCAAAGCT	97
	Rv: CCGCTCTTCACTCTCTTCCA	
CXCL14	Fw: CTACAGCGACGTGAAGAAGC	84
	Rv: ACGCTCTTGGTGGTGATGAT	
ICAM1	Fw: GTGACCGTGAATGTGCTCTC	82
	Rv: CCTGCAGTGCCCATTATGAC	
B-ACTIN	Fw: GAGCTACGAGCTGCCTGACG	122
	Rv: GTAGTTTCGTGGATGCCACAG	

Table 3. Selection of the 34 most differentially expressed genes (17 more expressed in SAC and 17 in hmMSI-H) as obtained from the array analysis.

Gene ID	Gene description	>expressed in	raw.p.value	adj.p.value
FANK1	fibronectin type III and ankyrin repeat domain 1	SAC	7,49E+07	0.0005
GTF2IRD2	GTF2I repeat domain containing 2	SAC	9,53E+06	0.0006
MTMR8	myotubularin related protein 8	SAC	9,93E+07	0.0006
TMEM8B	transmembrane protein 8B	SAC	1,58E+08	0.0008
FRZB	frizzled related protein	SAC	2,37E+08	0.0011
IYD	iodotyrosine deiodinase	SAC	2,71E+08	0.0011
GPR56	adhesion G protein-coupled receptor G1	SAC	3,25E+08	0.0013
KCNMB3	K+ Ca2+-activated channel subfamily M regulatory β3	SAC	3,36E+08	0.0016
PLEKHG3	pleckstrin homology and RhoGEF domain containing G3	SAC	4,98E+08	0.0017
CRCP	calcitonin gene-related peptide-receptor component protein	SAC	5,25E+06	0.0017
TUFT1	tuftelin 1	SAC	5,57E+08	0.0018
ZNF140	zinc finger protein 140	SAC	6,32E+08	0.0019
CDK20	cyclin dependent kinase 20	SAC	6,35E+08	0.0019
IGFBP2	insulin like growth factor binding protein 2	SAC	6,84E+08	0.0020
AXIN2	axin 2	SAC	7,29E+08	0.0020
CDK6	cyclin dependent kinase 6	SAC	8,09E+06	0.0021
CXCL14	C-X-C motif chemokine ligand 14	SAC	8,22E+08	0.0021
GPR109B	hydroxycarboxylic acid receptor 3	hmMSI-H	3,04E+07	0.0003
LILRB3	leukocyte immunoglobulin like receptor B3	hmMSI-H	2,83E+07	0.0003
CD300A	CD300 glycoprotein involved in immune response	hmMSI-H	2,78E+07	0.0003
SLC11A1	solute carrier family 11 member 1	hmMSI-H	2,74E+06	0.0006
GPR84	G protein-coupled receptor 84	hmMSI-H	2,42E+07	0.0003
CLEC4D	C-type lectin domain family 4 member D	hmMSI-H	2,10E+07	0.0002
CD14	CD14 molecule involved in innate immune response	hmMSI-H	2,07E+07	0.0002
FCGR2B	Fc fragment of IgG receptor IIb	hmMSI-H	1,75E+07	0.0002
CCL3L3	C-C motif chemokine ligand 3 like 3	hmMSI-H	1,72E+07	0.0006
SOD2	superoxide dismutase 2	hmMSI-H	1,30E+07	0.0002
ICAM1	intercellular adhesion molecule 1	hmMSI-H	1,12E+07	0.0002
MCHR1	melanin concentrating hormone receptor 1	hmMSI-H	1,02E+07	0.0002
CSF3R	colony stimulating factor 3 receptor	hmMSI-H	5,69E+05	0.0001
FPR2	formyl peptide receptor 2	hmMSI-H	5,25E+06	0.0001
SIGLEC5	sialic acid binding Ig like lectin 5	hmMSI-H	4,77E+06	0.0001
CLEC4A	C-type lectin domain family 4 member A	hmMSI-H	2,37E+06	0.0001
LILRA3	leukocyte immunoglobulin like receptor A3	hmMSI-H	2,11E+06	0.0001

SAC: Serrated adenocarcinoma; hmMSI-H: Colorectal carcinoma showing molecular and histological features of microsatellite instability, For simplification purposes, pseudogenes and non-coding RNA were excluded from this table. Genes chosen for validation by qPCR and IHC are written in bold letters

Table 4. Immunohistochemical expression of CRCP, CXCL14 and ICAM proteins in SAC and hmMSI-H CRC.

Protein	Tumor type	Negative n (%)	Positive n (%)	p-value
CRCP	SAC	22 (55)	18 (45)	
	hmMSI-H	10 (50)	10 (50)	p=0.4628
CXCL14	SAC	26 (65)	14 (35)	
	hmMSI-H	18 (90)	2 (10)	p=0.0353
ICAM1	SAC	22 (50)	18 (45)	
	hmMSI-H	5 (25)	15 (75)	p=0.0194

SAC: Serrated adenocarcinoma; hmMSI-H: Colorectal carcinoma showing molecular and histological features of microsatellite instability.

Figure legends

Figure 1. Terms of Gene Ontology Biological Processes differentially represented between SAC and hmMSI-H. A. The scatterplot shows the remaining biological processes which are differentially expressed between SAC and hMSI-H after the redundancy reduction. The graph is represented in a two dimensional space derived by applying multidimensional scaling to a matrix of the GO terms' semantic similarities [93]. B. The Tag Cloud displayed words which are differentially represented from the SAC vs. hMSI-H comparison with larger and darker letters signifying stronger overrepresentation. Underrepresented keywords are not displayed in the Tag Cloud.

Figure 2. qPCR results of the mRNA expression of DIO3 and FOXD2 genes in SAC and CC tumoral tissue as well as in adjacent non-tumoral specimens. * indicates statistical significance.

Figure 3. Immunohistochemical expression of CRCP, CXCL14 and ICAM1 in normal colorectal mucosa. Original 20X magnification.

Figure 4. Immunohistochemical expression of CRCP, CXCL14 and ICAM1 in serrated adenocarcinoma (SAC) and CRC showing histological and molecular features of

microsatellite instability (hmMSI-H). Original 20X magnification in all except in ICAM1 in SAC (X40).

Supplementary material

Supplementary material 1. Differentially expressed KEGG pathways and Gene Ontology terms in the comparison between SAC and hmMSI-H transcriptomic analysis (p-adjusted <0.05)

Supplementary material 2. Paintomics representations of KEGG pathways A. Cytokine-cytokine receptor interactions illustrating differences in the contribution of specific cytokines and certain cytokine families (mainly, CXC chemokines) in each type of CRC subtype. B. Colorectal cancer pathway which shows an over-representation of TGFB1, PIK3CG, RAC2 and BIRC5 activities in SAC and those of MAPK10, AXIN2, TCF7 and mismatch repair proteins MLH1 and MSH2 in hmMSI-H. C. Antigen processing and presentation panel illustrates a over-expression in hmMSI-H of genes whose proteins are involved in antigen processing through MHC class II and, especially class I. Proteins whose gene expression was higher in SAC are depicted in blue boxes those higher in hmMSI-H cases are represented in red boxes, the intensity of the colour indicating the strength of this difference [92]