MMP-7 and *SGCE* as distinctive molecular factors in sporadic colorectal cancers from the mutator phenotype pathway

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Abstract. Colorectal cancers (CRCs) from the suppressor and the mutator carcinogenic pathways display distinctive pathological and clinical features that remain not completely understood. In this context, the aim of this work was to study the differential expression of metalloproteinases and adhesion molecules related to cancer invasiveness in both groups of tumours. We analyzed 84 tissue specimens, 42 primary sporadic CRCs obtained from patients who underwent radical surgery, and its corresponding control tissues. According to microsatellite instability, 31 cancers showed low or null microsatellite instability (MSI-L/MSS) and 11 tumours displayed high microsatellite instability (MSI-H). Expression assays were established using the Oligo GEArray® human extracellular matrix and adhesion molecules microarray containing 114 genes. Real-time quantitative PCR (RT-qPCR) confirmed expression data from arrays, using TaqMan probes. Results from oligoarray expression analyses indicated that *ITGA3*, *ITGA9*, *ITGB4*, *ITGB7* and *MMP15* had significantly higher expression levels in MSI-H tumours versus MSS/MSI-L cancers, whereas *COL12A1*, *CSPG2*, *FN1*, *MMP-7* and *SGCE* were down-regulated in tumours with high microsatellite instability when compared to the stable group. After RT-qPCR validation, two of these genes, *MMP-7* and *SGCE*, were confirmed to have statistical differences between the two groups of tumours studied. In both cases, MSI-H tumours displayed significant lower expression levels than MSI-L/MSS tumours. In conclusion, these two distinctive molecular markers could be related to a diminished invasion in colorectal tumours from the mutator pathway, this may contribute to the understanding of the better patient prognosis conferred by this type of tumours.

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Introduction

Two major carcinogenic pathways have been described in colorectal cancer (CRC). Suppressor phenotype, that constitutes 80% of sporadic CRCs, was described in detail by Fearon and Vogelstein in 1990 (1). Mutator phenotype is the other main pathway of colorectal carcinogenesis and represents 15-20% of sporadic CRCs. In this case, genes of the mismatch repair (MMR) system suffer alterations that affect their role in the repair of DNA base mismatches. *MLH1* is the MMR gene most frequently altered, usually down-regulated by promoter hypermethylation (2,3). Primary alterations in MMR genes cause a huge accumulation of mutations in microsatellite sequences, short-tandem repeat sequences widespread all along the genome. Some of these microsatellite mutations occur in encoding or regulation sequences of important genes, such as *TGFβRII*, *MSH3*, *MSH6*, or *TCF4* (4). A panel of five microsatellite markers defined by the National Cancer Institute (5) has been established in order to classify a tumour as microsatellite stable or instable.

Besides the molecular differences between these two groups, distinctive pathological and clinical characteristics have been described. Thus, microsatellite instability-high (MSI-H) tumours tend to locate in the right side of the colon, have lymphocytic infiltration, mucin secretion and poor differentiation. It is also well-established that MSI-H tumours show better prognosis (6). Molecular explanations for this better prognosis are not fully understood yet.

Matrix metalloproteinases (MMPs) are involved in extracellular matrix (ECM) remodelling. Breaking the ECM provides the key for cellular invasion, and it is one of the major facts for metastasis (7). Interestingly, recent data also confer MMPs antitumour properties. For example MMP-8 prevents metastasis through a reduction of invasiveness and an increasement of cell adhesion (8). MMPs are a family of zinc-dependent enzymes synthesized as proenzymes. Their activity in normal steady-states tissues is low, but controlled

by cytokines, growth factors, tissue inhibitors of metalloproteinases (TIMPs) and α_2 macroglobulin, among others (9). On the other hand, molecules such as integrins and cadherins are also related to adhesion and MMP regulation (7). MMP-3 and MMP-7 have E-cadherin as a target for proteolysis (10). It has been also seen that α3ß1 integrin is able to induce mRNA stability of *MMP-9* (11) and αß1 integrin also regulates expression of other MMPs such as MMP-1, -2, -3, -13 and -14 (12). Apart from degrading ECM components (i.e. collagens, fibronectin, laminin), MMPs can induce the release of factors that increase cell proliferation as insulin-like growth factor (IGF), fibroblast growth factor (FGF); or that inhibit that cell proliferation as tumour growth factor-ß (TGFß). They can also mediate angiogenesis through αvß3 integrin or interfere in cell survival with Fas shedding.

However, all the biochemical factors involved in invasion and metastasis are not fully elucidated. In previous works, we analyzed E-cadherin status, Wnt pathway factors and levels and activity of MMP-3, MMP-9 and MMP-2 in CRCs from the suppressor and the mutator tumourigenesis pathways (13-15). Thus, we found molecular differences that may contribute to explain the divergent patient prognosis in both types of CRCs. In this context we decided to study the expression of different extracellular matrix and adhesion molecules in tumours from patients affected by sporadic colorectal cancer with and without high microsatellite instability.

Materials and methods

Patients and tissue samples. We analyzed 84 colorectal tissues, corresponding to 42 tumour samples and their corresponding non-tumoural pairs, obtained from patients with primary sporadic colorectal cancer that underwent surgery at San Carlos Hospital of Madrid. Prior to surgery, none of these patients had received neoadjuvant therapy. Informed consent was obtained from patients prior to investigation and this study was approved by the Ethics Committee from the Hospital. After surgical resection, all tissue samples were snap-frozen in liquid nitrogen and stored at -80˚C until processed. Cryostat sectioned, H&E stained samples from each tumour block were examined microscopically by two independent pathologists to confirm the presence of >80% tumour cells. Paired normal tissues from the same patient, used as controls, were obtained at least 10 cm away from the margin of the tumour and microscopically confirmed.

Extracellular matrix and adhesion molecules expression assays. This study was established for 42 sporadic CRCs and its corresponding control samples in which we had previously determined the MSI status. Genomic DNA from tumour and control samples was isolated as described by Blin and Stafford (16). Tumour samples were classified according to microsatellite instability status following previous established criteria (5). Thus, 31 tumours were classified as MSS/MSI-L and 11 as MSI-H. These cases were selected from a larger CRC population previously analyzed in order to have a sufficient number of MSI-H tumours, since this CRC type is not that abundant. For expression assays, RNA was extracted from tissue samples by TRIzol® method (Invitrogen™ , California, USA) according to manufacture's indications. Then, using Oligo GEArray human extracellular matrix and adhesion molecules microarray (OHS-013, SABiosciences™ , Frederick, USA) containing 114 genes, this RNA was amplified; biothin marked and hybridized to the corresponding oligonucleotide membranes. The analysis of these membranes was developed by the GEArray analysis expression suite software (SABiosciences). *GAPDH* was performed as a house-keeping gene and was included in the membranes. We also evaluated the expression profile of a pool obtained from the corresponding normal samples.

Expression data from arrays were confirmed investigating gene expression by real-time quantitative PCR (RT-qPCR), using TaqMan probes (MGB 'Assay on Demand' probes were purchased to Applied Biosystems, New Jersey, USA), following the manufacturer's instructions. Genes selected for RT-qPCR analyses were those with at least ± two-fold differences of expression between the two tumour types considered in this work. The comparative threshold cycle (Ct) method was used to calculate the relative gene expression. For quantification of gene expression, the target genes values were normalized to the expression of the endogenous reference (*GAPDH*). Thus, the amount of target gene, normalized to *GAPDH* and relative to a calibrator (normal pool expression) is given by RQ, being $RQ = 2^{-\Delta\Delta Ct}$ [$\Delta\Delta Ct$ = ΔCt for any sample - ΔCt for the calibrator; ΔCt = Ct (target gene) - Ct (*GAPDH*)].

Statistical analysis. The SPSS version 11.5 software was used for the statistical analyses. T-student or Mann-Whitney tests were selected for studying differences between groups whether they behave as normal distribution or not. Linear regression and correlation analyses for the strength of association between variables were evaluated through Pearson's coefficient correlation regarding to a normal distribution data. ANOVA studies were selected when three or more variables were evaluated, checking previously normal distribution and homocedasticity. For RT-qPCR data, all statistical analysis were performed with $\Delta\Delta Ct$ values, because the RQ values do not follow a linear progression.

Results

DNA samples from patients affected by sporadic colorectal cancer were classified on basis of their microsatellite instability status. Expression profiles analyses were performed in all these tumour samples and in a pool of the corresponding normal tissues. The oligonucleotide microarray for extracellular matrix and adhesion molecules included 114 genes plus others suitable for a house-keeping gene use (Fig. 1). We sought for differences between tumours with and without high microsatellite instability. The limit was established in ±2 times of difference of expression. Table I shows the genes that had the major differences after the array analysis. Five of them had higher level of expression in MSI-H tumours *versus* MSS/MSI-L tumours: *ITGA3* (integrin α 3) 3.19-fold increase, *ITGA9* (integrin α 9) 3.99-fold increase, *ITGB4* (integrin ß 4) 2.87-fold increase, *ITGB7* (integrin ß 7) 3.10-fold increase, MMP15 (matrix metalloproteinase 15) 2.85-fold increase. On the other hand,

A)

	A	B	C	D	E	F	G	H
1	RPS27A	ADAMTS1	ADAMTS13	ADAMTS8	CD44	CDH1	CNTN1	COL11A1
2	COL11A2	COL12A1	COL14A1	COL15A1	COL16A1	COL18A1	COL19A1	COL1A1
3	COL24A1	COL27A1	COL4A2	COL4A3	COL4A6	COL5A1	COL5A3	COL6A1
4	COL6A2	COL6A3	COL7A1	COL8A1	COL8A2	COL9A1	VCAN	CTGF
5	CTNNA1	CTNNB1	CTNND1	CTNND2	ECM1	FN1	HAS1	ICAM1
6	ITGA1	ITGA10	ITGA11	ITGA2	ITGA2B	ITGA3	ITGA4	ITGA5
7	ITGA6	ITGA7	ITGA8	ITGA9	ITGAL	ITGAM	ITGAV	ITGAX
8	ITGB1	ITGB2	ITGB3	ITGB4	ITGB5	ITGB6	ITGB7	ITGB8
9	KAL1	LAMA1	LAMA2	LAMA3	LAMA4	LAMA5	LAMB1	LAMB2
10	LAMB3	LAMB4	LAMC1	MMP1	MMP10	MMP11	MMP12	MMP13
11	MMP14	MMP15	MMP16	MMP17	MMP2	MMP20	MMP24	MMP26
12	MMP3	MMP7	MMP8	MMP9	NCAM1	PECAM1	SELE	SELL
13	SELP	SGCE	SPARC	SPG7	SPOCK1	SPP1	TGFBI	THBS1
14	THBS2	THBS3	THBS4	TIMP1	TIMP2	TIMP3	CLEC3B	TNC
15	VCAM1	VTN	PUC18	Blank	Blank	**AS1R2**	**AS1R1**	**AS1**
16	**GAPDH**	**B2M**	**HSP90AB1**	**HSP90AB1**	**ACTB**	**ACTB**	**BAS2C**	**BAS2C**

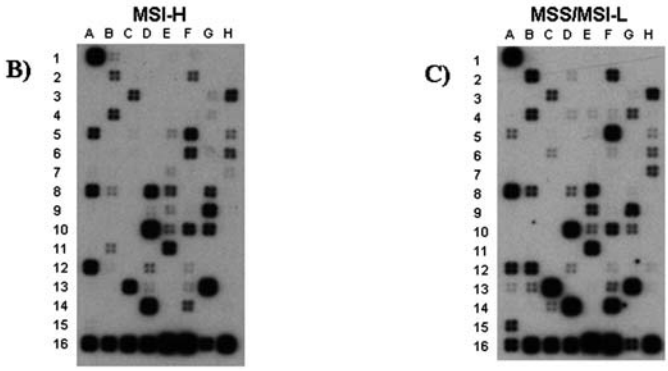

Figure 1. (A) Genes included in expression analyses by arrays. (B) and (C) Representative array results of the two CRC subgroups established in function of MSI status. (B) Tumours showing high microsatellite instability (MSI-H). (C) Tumours showing low or null microsatellite instability (MSI-L/MSS).

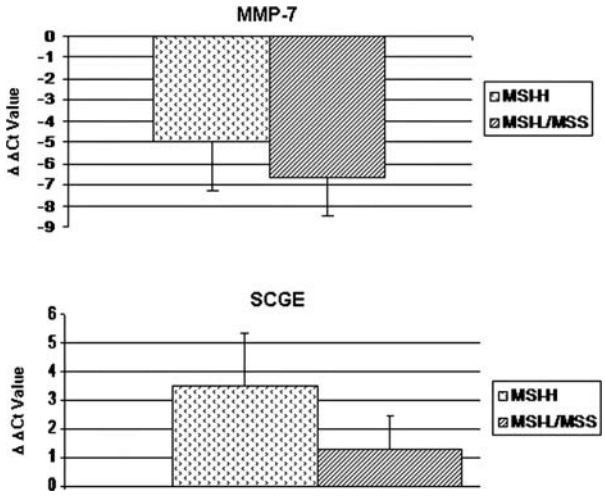

Figure 2. *MMP-7* and *SGCE* expression differences by RT-qPCR between CRCs with and without high microsatellite instability. Values are shown as ΔΔCt. *SGCE* has lower values of this parameter in tumours MSI-L/MSS, that means higher expression of this gene. *MMP-7* is represented with negative values of ΔΔCt that portray higher levels of expression of the samples compared to the calibrator, non-tumoural samples in our case. So the more negative the ΔΔCt is, the lower the value of ΔCt sample, which means the higher level of expression. Then *MMP-7* is more up-regulated in tumours MSI-L/MSS than in unstable tumours.

five genes were down-regulated in tumours with instability in microsatellites when compared to stable tumours: *COL12A1* (collagen type XII, α 1) 0.45-fold decrease, *CSPG2* (Versican) 0.39-fold decrease, *FN1* (fibronectina 1) 0.36-fold decrease, *MMP-7* (matrix metalloproteinase 7) 0.22-fold decrease, *SGCE* (sarcoglycan, ε) 0.39-fold decrease.

To validate expression data from microarray analyses, we used RT-qPCR technique that allowed us to quantify levels of mRNA in all cases. RT-qPCR is a commonly used validation tool for confirming gene expression results obtained from microarray analysis. Thus, RT-qPCR confirmed two genes to have statistical difference between tumours showing high microsatellite instability (MSI-H) and those classified in the MSI-L/MSS group (Fig. 2). Thus, *SGCE* [MSI-H: 3.55 (0.53) as mean (standard error)]; {2.36, 4.74} as confidence interval and MSI-L/MSS 1.25 (0.19) as mean (standard error); {0.36, 1.64} as confidence interval) and *MMP-7* [MSI-H: -4.97 (0.70) as mean (standard error)]; {-6.52, -3.41} as confidence interval and MSI-L/MSS -6.8 (0.31) as mean (standard error); {-7.45, -6.16} as confidence interval) had significant differences ($P<0.05$) that proved lower levels of expression in tumours MSI-H *versus* tumours MSS/MSI-L. These genes and others are detailed in Table II. The values for Fig. 2 and Table II are given as ΔΔCt, higher value of these data indicates lower expression. *MMP-7* is up-regulated in both types of tumours according to the RQ value (RQ MSI-H tumours = 31.28; RQ MSI-L/MSS = 104.42), which implies normalization with normal tissues. However, as seen in Fig. 2, *MMP-7* expression is much higher in MSS/MSI-L tumours than in MSI-H tumours according to the lower value of ΔΔCt in stable tumours. Concerning *SGCE*, microsatellite stable tumours had lower levels of mRNA than non-tumoural

Table I. Gene expression screening by extracellular matrix and adhesion molecules expression oligoarrays: significant differences between CRCs with and without MSI-H.

Gene symbol	GeneBank reference	Name	Function	MSI status	Expression values mean (mean ± SE)	P-value
ITGA3	NM_002204	Integrin, α 3	Cell surface adhesion molecule that join with ß 1 subunit to form an integrin that interacts with many extracellular-matrix proteins.	MSI-L/MSS MSI-H	0.374±0.080 1.185±0.471	0.018
ITGA9	NM_002207	Integrin, α 9	The protein encoded by this gene, when bound to the ß 1 chain, forms an integrin that is a receptor for VCAM1, cytotactin and osteopontin.	MSI-L/MSS MSI-H	0.020±0.004 0.076±0.034	0.014
ITGB4	NM_000213	Integrin, ß 4	This gene encodes the integrin ß 4 subunit, a receptor for the laminins and associate with α 6 subunit.	MSI-L/MSS MSI-H	0.629±0.083 1.811±0.865	0.003
ITGB7	NM_000889	Integrin, ß 7	The ß 7 integrin is expressed by leukocyte subsets and mediate interactions of these cells with extracellular matrix molecules as well as with endothelial and epithelial cells.	MSI-L/MSS MSI-H	0.252±0.072 0.778±0.217	0.016
MMP15	NM_002428	Matrix metallo-proteinase 15	This gene is a member of the membrane-type matrix metalloproteinase (MT-MMP) subfamily.	MSI-L/MSS MSI-H	0.110±0.031 0.314±0.083	0.044
COL12A1	NM_004370	Collagen, type XII, α 1	Type XII collagen is a homotrimer found in association with type I collagen, this modifies the interactions between collagen I fibrils and the surrounding matrix.	MSI-L/MSS MSI-H	0.694±0.134 0.314±0.100	0.036
CSPG2	NM_004385	Versican	Versicanis a large chondroitin sulfate proteoglycan of the extracellular matrix which interacts with hyaluronan at the N-terminal G1 domain.	MSI-L/MSS MSI-H	0.380±0.117 0.188±0.105	0.034
FN1	NM_002026	Fibronectin 1	Fibronectin is involved in cell adhesion and migration processes. In a soluble dimeric form is present in plasma, and in a dimeric or multimeric form at the cell surface and in extracellular matrix.	MSI-L/MSS MSI-H	1.675±0.724 0.607±0.131	0.038
MMP7	NM_002423	Matrix metallo-proteinase 7	The enzyme encoded by this gene degrades proteoglycans, fibronectin, elastin and casein and differs from most MMP family members in that it lacks a conserved C-terminal protein domain.	MSI-L/MSS MSI-H	1.557±0.867 0.350±0.103	0.054
SGCE	NM_003919	Sarcoglycan, ε	Sarcoglycans are transmembrane components in the dystrophin-glycoprotein complex which help stabilize the muscle fiber membranes and link the muscle cytoskeleton to the extracellular matrix.	MSI-L/MSS MSI-H	0.332±0.152 0.127±0.062	0.059

Table II. Extracellular matrix and adhesion molecules expression differences between MSI-L/MSS and MSI-H colorectal tumors, after validation by RT-QPCR.[a]

Gene symbol	MSI status	Expression values		
		ΔΔCt mean (mean ± SE[b])	Confidence interval	P-value
ITGA3	MSI-L/MSS	1.94±0.14	1.65-2.24	0.250
	MSI-H	1.40±0.42	0.44-2.36	
ITGA9	MSI-L/MSS	1.22±0.38	0.33-2.11	0.409
	MSI-H	1.94±0.72	0.27-3.61	
ITGB4	MSI-L/MSS	0.53±0.11	0.29-0.77	0.397
	MSI-H	0.19±0.37	(-0.62)-1.01	
ITGB7	MSI-L/MSS	2.6±0.18	2.24-2.97	0.056
	MSI-H	1.84±0.40	0.93-2.74	
MMP15	MSI-L/MSS	2.15±0.37	1.23-3.07	0.173
	MSI-H	1.48±0.29	0.79-2.16	
COL12A1	MSI-L/MSS	(-1.95)±0.21	(-2.39)-(-1.52)	0.064
	MSI-H	(-1.14)±0.43	(-2.11)-(-0.17)	
CSPG2	MSI-L/MSS	(-0.51)±0.18	(-0.87)-(-0.14)	0.318
	MSI-H	0.03±0.49	(-1.05)-1.12	
FN1	MSI-L/MSS	(-0.05)±0.26	(-0.59)-0.49	0.123
	MSI-H	0.86±0.64	(-0.59)-2.30	
MMP7	MSI-L/MSS	(-6.8)±0.31	(-7.45)-(-6.16)	0.009
	MSI-H	(-4.97)±0.70	(-6.52)-(-3.41)	
SGCE	MSI-L/MSS	1.25±0.19	0.36-1.64	0.001
	MSI-H	3.55±0.53	2.36-4.74	

[a]High ΔΔCt values express lower levels of expression. [b]SE, standard error.

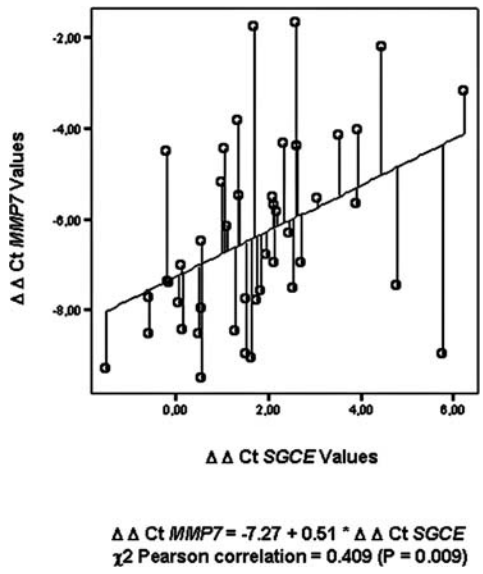

Figure 3. Significant correlation between *SGCE* and *MMP-7* expression by RT-qPCR in sporadic colorectal tumours.

samples (RQ MSI-L/MSS = 0.41). Microsatellite unstable tumours showed a higher decreased mRNA levels compared to non-tumoral samples (RQ MSI-H = 0.08) (Fig. 2). Of interest, levels of ΔΔCt of *MMP-7* and *SGCE* showed a lineal dependency (P=0.009) and had a Pearson's correlation factor of 0.409 (Fig. 3).

Discussion

Stable and unstable colorectal tumours develop through different ways, and therefore they confer different phenotypes. However, many clinical differences have been described between these two groups. The most important difference is the better clinical outcome in MSI-H tumours and there is not yet a clear molecular explanation for it. Searching for differential molecular alterations in both types of colorectal tumours might shed some light on their distinctive clinical behaviour.

In a previous work (13), we showed a significant different expression profile for genes involved in the Wnt pathway in sporadic colorectal cancers with and without microsatellite instability. The Wnt pathway has at least one activating mutation in any of the Wnt-related molecules in a 90% of cases (17). Our results showed that the expression of the Wnt-related genes in MSI-H tumours was similar to the expression of normal tissues. However, the following genes were down-regulated in MSI-L/MSS tumours: *PPP2R1B*

(protein phosphatase 2 regulatory subunit A, ß isoform), CSNK1D (casein kinase 1, δ), *KREMEN2* (kringle containing transmembrane protein 2), *DVL2* (dishevelled 2), *FBXW4* (F-box and WD repeat domain containing 4) and *TLE3* (transducin-like enhancer of split 3). Most of those genes have a regulatory function in the WNT pathway, so that might explain the differential regulation of the WNT pathway in colorectal tumours with and without microsatellite instability.

Moreover, a differential E-cadherin truncation in both types of tumours was found. Thus, E-cadherin had a higher level of inactivation in MSI-L/MSS tumours (13). E-cadherin binds ß-catenin to its cytoplasmic domain (18). The cleavage of E-cadherin could increase levels of ß-catenin in the cytoplasm leading to the activation of WNT pathway in tumours that lose E-cadherin in the membrane (19,20). In fact, we have detected higher levels of ß-catenin by immunohistochemistry in MSI-L/MSS tumours (13).

The WNT pathway can regulate metalloproteinases. Thus, *MMP-7* (21), *MMP-14* (22) and *MMP-26* (23) have TCF-4-binding sites in their promoter, so that it can be activated by this transcription factor through ß-catenin activation. In addition, *MMP-2* and *MMP-9* are induced by ß-catenin through their LEF/TCF-binding sites after WNT activation in T-cells (24). We studied the differential expression by oligo microarrays of several MMPs in tumours with or without microsatellite instability. *MMP-7* has been described to be up-regulated in colon and rectal tumours (25,26). We have shown in this work that *MMP-7* expression is significantly higher in MSI-L/MSS tumours as compared to MSI-H tumours. MMP-7 is an enzyme more likely expressed in C and D Dukes stages for CRC, and its implication in tumour invasion, metastasis, lymph node status and poor prognosis has been shown (21,27). *MMP-7* is a target gene of the WNT pathway and its expression is regulated by ß-catenin in CRC (21). Our finding that *MMP-7* is higher expressed in tumours without microsatellite instability would imply a higher activation of WNT pathway in this type of tumours. This is consistent with the overexpression of cyclin D1 in MSI-L/MSS tumours, another target gene of the WNT pathway (13). Our results suggest that MMP-7 is an important distinctive marker of colorectal tumours with or without microsatellite instability.

One striking point concerning MMP expression is our result of an increased expression of MMP-15 in MSI-H tumours *versus* MSS/MSI-L tumours obtained in the microarray assay. MMPs have been shown traditionally involved in ECM degradation, however recent data point to a possible protective effect of these enzymes in cancer (8).

SGCE is the ε isoform member of sarcoglycan family. Sarcoglycans are transmembrane components in the dystrophin-glycoprotein complex which help stabilize the muscle fiber membranes and link the muscle cytoskeleton to the extracellular matrix. Mutations in this gene have been mainly related to myoclonus-dystonia syndrome (MDS) and inherited movement disorder (28). However its involvement in cancer is not clear. Dong *et al* (29) defined an expression profile for human hepatocarcinoma. In this assay they showed that *SGCE* was overexpressed in these tumours in comparison with non-tumour liver. In addition, *SGCE* was demonstrated to be up-regulated in B-cell chronic lymphocytic leukaemia patients (30). On the other hand, these studies are merely descriptive. The mechanism of how this molecule could be involved in tumour development has not been described. Our results indicate an up-regulation of this gene in MSI-L/MSS tumours *versus* MSI-H. As the function of SGCE is unknown, it is difficult to explain the role of this gene in cancer. Taking this into account, we are not able to suggest possible causes or consequences of this differential expression. *SGCE* might be only a marker with no concrete function. Further studies are needed.

In conclusion, we report two molecular markers that differentiate tumours from the mutator pathway *versus* the suppressor pathway. Thus, colorectal tumours from the mutator phenotype pathway, with high microsatellite instability, displayed lower *MMP-7* expression levels as compared to MSI-L/MSS tumours. Moreover, colorectal tumours from the mutator phenotype pathway displayed lower *SGCE* expression levels as compared to MSI-L/MSS tumours. Thus, *MMP-7* could be related to the already described diminished invasion and/or aggressiveness in colorectal tumours from the mutator pathway, that may contribute to understanding the better patient prognosis conferred by this type of tumours. *SGCE* will need to be studied in depth to reach a more concrete conclusion.

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