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ORIGINAL ARTICLE

Toll-Like Receptor 4 and Matrix Metalloproteases 11 and 13 as Predictors of Tumor Recurrence and Survival in Stage II Colorectal Cancer

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Abstract

Current clinical-pathologic stratification factors do not allow clear identification of high-risk stage II colorectal cancer (CRC) patients. Therefore, the identification of additional prognostic markers is desirable. Toll-like receptor (TLR)-4 is activated during tumorigenesis and matrix metalloproteases (MMPs) are involved in invasion and metastasis. We aimed to evaluate the expression and clinical relevance of TLR4, MMP11 and MMP13 for patients with stage II CRC. Immunohistochemistry was used to study the expression of TLR4, MMP11 and MMP13 in 96 patients with stage II CRC. We measured the global expression and the expression by different cell types (tumor cells, cancer-associated fibroblasts (CAFs) and mononuclear inflammatory cells (MICs)). The potential relationship between expressions of factors and different prognostic variables were evaluated. Our results show significant relationships between either TLR4 expression by tumor cells and MMP11 expression by CAFs and high risk of tumor recurrence. In addition, the concurrence of age ≥ 75 years and the non-expression of MMP11 by CAFs identify a subgroup of patients with a good prognosis. Our results show that TLR4 expression by tumor cells and MMP11 expression by CAFs may to improve the identification of patients with stage II CRC with a high-risk of relapse.

Keywords Prognostic factor · Stage II colorectal cancer · Survival · TLR4 · MMP11 · MMP13

Introduction

Colorectal cancer (CRC) is responsible for over 500,000 deaths annually worldwide [1, 2]. TNM (tumor, node, metastasis) stage represents the most important prognostic factor in this neoplasia [3, 4]. About 30–40% of CRCs are diagnosed in absence of nodal (N0) and distant metastasis (M0) (as stage II tumors) [5]. According to the American Joint Committee on Cancer (AJCC), stage II colon cancer includes 3 categories, stages IIA (T3, N0), IIB (T4a, N0), and IIC (T4b, N0) [6].

Stage II CRC is generally considered as of good prognosis; however, 25% of these patients, and who underwent surgery alone, develop recurrence [7, 8]. For this reason, the decision of use adjuvant chemotherapy in patients with these stage II tumors is questionable [9–11]. After surgery, some benefit may be derived from chemotherapy, but this strategy requires treating many patients who are already cured. For this reason, the current international guidelines suggest that it should be limited to patients at high risk of progression. At present, high-risk patients are identified on the basis of several pathological parameters, including positive margins, pT4 stage, poorly differentiated tumor, lymphatic/vascular invasion and less than 12 lymph nodes harvested [8, 12, 13]; but these parameters have a low inter-observer reproducibility [14, 15]. In addition, considering that the incidence of early stage CRC is increasing as a result of screening process, there is the need of additional useful prognostic markers to detect patients with stage II CRC that could benefit from more aggressive therapy.

Toll-like receptors (TLRs) are type I integral transmembrane glycoproteins, which are activated during tumorigenesis by several components, such as bacterias and viruses, products of tissue damage and necrosis, among

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other signals [16, 17]. TLR-mediated signals which lead to the activation of nuclear factor-kB (NF-κB), promoting, then,mitogen-activated protein kinases and inflammation-associated cancer [16]. Tumor expression of TLR3, TLR4, TLR7 and TLR9, have been related to prognosis in stage II CRC [18]. Likewise, TLRs have been related with several mechanisms in CRC, such as colon cancer cell adhesion [19], tumorigenesis and tumor progression [20]; but also with the inhibition of cell proliferation, promotion of apoptosis and improvement of anti-tumor effects of radiotherapy and chemotherapy [21]. Recently, we found that TLR4 expression by cancer cells was associated with a lower tumor recurrence, whereas the expression by cancer-associated fibroblasts (CAFs) was associated with a high rate of tumor recurrence and with a shortened overall survival in an overall population of patients with CRC [22].

One key aspect in tumor invasion and metastasis is the degradation of the stromal connective tissue and basement membrane components. However, some of their matrix components, such as interstitial collagens, are very resistant to attack of proteolytic enzymes, being degraded only by matrix metalloproteases (MMPs) [23]. MMPs are also able to influence tumor cell progression as a consequence of their ability to cleave cell surface receptors, cell adhesion molecules, growth factors or chemokines/cytoquines [24–27], and proapoptotic factors [28]. In addition, high MMPs expression by tumors have been related with poor prognosis in patients with various types of cancer [29–32].

Several studies have revealed that expression of MMPs and tissue inhibitors of MMPs (TIMPs) is higher in CRCs than in normal colorectal tissue [33, 34]. The prognostic relevance of these enzymes in CRC, for example MMP1, 7, 9, 13, and 14, and TIMP-1 and 2, also have been revealed [35–40]. Likewise, we found that MMP11 expression by CAFs and MMP13 by tumor cells were associated with poor prognosis in an overall population of patients with CRC [41].

The aim of the present work was to evaluate the expression of TLR4, MMP11 and MMP13 as prognostic markers in patients with stage II CRC.

Patients and Methods

Patients' Selection, Patients' Characteristics, and Tissue Specimen Handling

This study included 96 patients with CRC (age range: 46–87 years; median age: 68.5 years) (Table 1). Forty five surgically resected pTNM stage II (24 pT3N0M0, 14 pT4aN0M0 and 7 pT4bN0M0) CRCs, which underwent disease progression within five years since the initial diagnosis, were taken from our cancer registry and included in the present study (16 with local recurrence, 10 with carcinomatosis, 24 with distant metastasis, and 5 with both local and distance recurrence), and 28 of them died. Then, 51 stage II CRCs with no evidence of disease progression in a follow-up time longer than five years were selected.

Table 1 Basal characteristics of 96 patients with colorectal cancer II stage

Characteristics	Without recurrence No. (%)	With recurrence No. (%)
All patients	51	45
Age (years)		
≤68.5	29 (56.9)	19 (42.2)
>68.5	22 (43.1)	26 (57.8)
Sex		
Male	25 (49.0)	21 (46.7)
Female	26 (51.0)	24 (53.3)
Tumor size		
T3	42 (82.4)	24 (53.3)
T4a	8 (15.7)	14 (31.1)
T4b	1 (2.0)	7 (15.6)
Histological grade*		
Well differentiated	13 (25.5)	12 (26.7)
Moderately differentiated	36 (70.6)	32 (71.1)
Poorly differentiated	1 (2.0)	0
Type of recurrence		
Local	–	21 (46.7)
Carcinomatosis	–	10 (22.2)
Distance	–	29 (64.4)
Hepatic metastasis	–	16 (35.6)
Lung metastasis	–	11 (24.4)
Brain metastasis	–	1 (2.2)
Bone metastasis	–	1 (2.2)
Death	0	28 (62.2)
Adyuvant treatment		
No	51 (100.0)	39 (86.7)
Yes	0	6 (13.6)
Tumor location		
Right colon	20 (39.2)	16 (35.6)
Transverse colon	5 (9.8)	2 (4.4)
Left colon	26 (51.0)	26 (57.8)
Right and left colon	0	1 (2.2)

*1 case unknown without recurrence and 1 case unknown with recurrence

In all cases, the standard operation is en-bloc resection of the colon cancer with regional lymphadenectomy and margins were at least 10 cm proximal and 5 cm distal to the tumor. Pathological staging had been performed according to the seventh edition of pTNM system and at least 12 lymph nodes had been retrieved from the peri-visceral adipose tissue. Only 6 patients received chemotherapy and all of them underwent disease progression.

After surgical resection, all tissues were processed for pathologic examination and samples were removed from the

tumors, avoiding grossly necrotic tissues. Samples used in this study have a low and similar level of desmoplastic reaction. The study adhered to national regulations and was approved by our institution's Ethics and Investigation Committee.

Tissue Array Immunohistochemistry

Tumor specimens were fixed in 10% neutral buffered formalin and after being embedded in paraffin. After to define histopathologically representative tumor areas on hematoxylin and eosin-stained sections, tumor tissue array (TA) blocks were obtained by using a manual tissue arrayer (Beecher Instruments, Sun Prairie, WI, USA), as described elsewhere [42]. Four composite high-density TA blocks were performed, which include two cores for each case, and serial 5-μm sections,, were consecutively cut in a microtome (Leica Microsystems, Wetzlar, Germany) and transferred to adhesive-coated slides. One section from each TA block, was used to confirm that the sample was representative of the original tumor by staining with hematoxylin and eosin. Tissue sections were treated in a PTLink (Dako, Glostrup, Denmark) at 96 °C for 20 min, in citrate buffer of pH 6.1 for TLR4 and in Tris/EDTA buffer of pH 9 for MMP13. Immunohistochemistry was performed using a TechMate TM50 autostainer (Dako). Antibodies for TLR4 (sc-10,741, 1/40, 45 min) was obtained from Santa Cruz (Santa Cruz Biotechnology, CA) and antibodies for MMP11 (MA5–11234, 1/1000, 60 min) and MMP13 (MA5–14238, 1/100, overnight) were obtained from ThermoFisher (Lab Vision, Fremont, CA, USA). Dilutions were made in Antibody Diluent (Dako) and antibodies were incubated at room temperature.

The slides were incubated in peroxidase-blocking solution (Dako) for 5 min., for to block endogenous peroxidase activity. As staining detection system, the EnVision Detection Kit (Dako) was used, and the sections were counterstained with hematoxylin, dehydrated with ethanol, and permanently coverslipped.

Immunohistochemical Analysis

For each evaluated protein, its immunoreactivity location, the percentage of stained cells and intensity were determined, as described previously [43]. The histological examination was performed by a pathologist (LOG) who blinded to the clinical outcome of the patients. All the cases were semiquantified for each protein-stained area, and by using an image-analysis system with the Olympus BX51 microscope and analysis software (analySIS; Soft Imaging System, Münster, Germany), as described before [44–46]. Briefly, each core was scanned with a 400X power objective in two different fields containing the with the computer program. The stained areas were selected, evaluated, and the final area ratio was the average of the two fields, as was described elsewhere [44–46]. The immunostaining intensity was evaluate by using a numeric score, as follows: 0, no staining; 1, weak staining; 2, moderate staining; and 3, intense staining. After the global mean score was obtained by multiplying the intensity score (*I*) by the percentage of stained cells (*PC*) (total score: *I x PC*). The mean score of two core biopsies was calculated for each tumor. The score of the paired tumor core was given when there was no tumor in a particular core..

In addition, the expert pathologist evaluated the immunohistochemical staining by each main cell type (tumor cells, CAFs and mononuclear inflammatory cells (MICs)). Stromal cells were distinguished from tumor cells because the latter are larger in size and while they are closely packed and arranged forming either acinar or trabecular patterns, stromal cells are spread. In addition, CAFs are spindle shaped, whereas MICs are small round cells.

Statistical Analysis

χ^2 test was used to calculate differences between percentages. Mann-Whitney or Kruskal-Wallis tests were used to compare immunostaining score values for each protein, which expressed as median (range). Cox univariate method was used for metastasis-free survival analysis. The PASW Statistics 18.0 software (SPSS Inc., Chicago, IL) was used for all calculations. $P \leq 0.05$ was considered as significant.

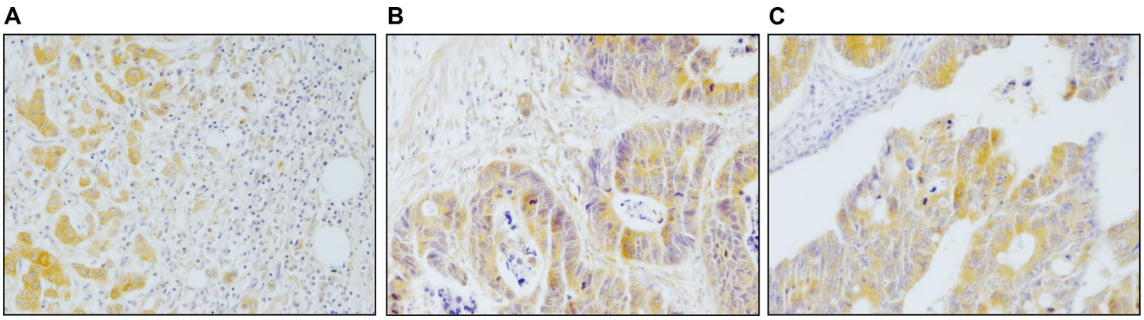

Fig. 1 Representative pictures of proteins immunostaining in colorectal tumors. Immunostaining for **a** Toll-like receptor 4 (TLR4), **b** Matrix Metalloprotease 11 (MMP11) and **c** Matrix Metalloprotease 13 (MMP13)

Table 2 Relationship between TLR4, MMP11 and MMP13 immunostaining *score* values and clinicopathological characteristics of 96 patients with colorectal cancer on stage II

Characteristics	Score values median (range) TLR4	Score values median (range) MMP11	Score values median (range) MMP13
Age	p = 0.010		
≤ 68.5 years	96.5	113.51	72.31
	(0–194)	(32–293)	(0–149)
≥ 68.5 years	117.43	126.12	80.1
	(0–240)	(0–254)	(0–179)
Sex			
Male	109.08	116.10	81.36
	(0–240)	(0–256)	(0–179)
Female	98.70	132.09	71.88
	(0–215)	(39–293)	(0–173)
Tumor size	p = 0.004		
T3	107.64	118.82	80.73
	(0–240)	(0–284)	(0–173)
T4a	92.37	114.71	58.50
	(0–191)	(46–293)	(0–180)
T4b	94.27	141.25	62.59
	(0–211)	(39–255)	(0–146)
Histological grade			
Well differentiated	105.54	109.56	81.36
	(0–210)	(0–226)	(0–179)
Moderately differentiated	106.36	126.85	72.99
	(0–240)	(32–284)	(0–158)
Poorly differentiated	0	293.05	0
		(0–293)	
Recurrence			
No	103.60	109.04	67.66
	(0–240)	(0–293)	(0–150)
Yes	110.37	126.48	80.10
	(0–216)	(39–284)	(0–179)
Tumor location			
Right colon	97.93	117.66	73.67
	(0–240.62)	(36–219)	(0–173)
Transverse colon	117.27	120.03	119.04
	(15–186)	(53–158)	(0–138)
Left colon	107.79	127.19	71.88
	(0–216)	(0–293)	(0–179)
Right and left Colon	134.16	100.58	91.64
	(134.16)	(100.58)	(91.64)

Results

TLR4, MMP11 and MMP13 Expression and Clinicopathological Characteristics of Patients and Tumors

In this study, we investigated expression levels of TLR4, MMP11 and MMP13, by immunohistochemical analysis of 96 stage II CRCs. Immunohistochemical staining shows a membrane location of TLR4 in positive cells, whereas MMP11 and MMP13 are located in the cytoplasm, being tumor cells the predominant positive cell type (Fig. 1).

The relationship between global expression (score values) of each factor, which ranged widely, and clinicopathological characteristics of patients and CRCs was analyzed. No significant association was found with sex of patients, histological

Table 3 Relationship between the cell type expressing TLR4, MMP11, MMP13, and clinicopathological characteristics in 96 patients with Colorectal Cancer stage II

	TLR4			MMP11			MMP13		
	TC	CAF	MIC	TC	CAF	MIC	TC	CAF	MIC
Age									
≤68.5 years	40 (43%)	9 (9%)	11 (11%)	46 (49%)	27 (29%)	13 (13%)	36 (39%)	3 (3%)	13 (14%)
≥68.5 years	45 (48%)	14 (15%)	12 (13%)	46 (49%)	23 (24%)	6 (6%)	40 (43%)	4 (4%)	13 (14%)
Sex									
Male	42 (45%)	10 (10%)	12 (13%)	42 (45%)	23 (24%)	10 (10%)	38 (41%)	5 (5%)	15 (16%)
Female	43 (46%)	13 (14%)	11 (11%)	50 (53%)	27 (29%)	9 (9%)	38 (41%)	2 (2%)	11 (11%)
Tumor size									
T3	63 (66%)	17 (18%)	16 (17%)	62 (65%)	31 (32%)	14 (15%)	55 (57%)	4 (4%)	20 (21%)
T4a	16 (17%)	5 (5%)	6 (6%)	22 (23%)	14 (15%)	4 (4%)	15 (16%)	2 (2%)	3 (3%)
T4b	6 (6%)	1 (1%)	1 (1%)	8 (8%)	5 (5%)	1 (1%)	6 (6%)	1 (1%)	3 (3%)
Histological grade									
Well differentiated	26 (28%)	6 (6%)	8 (8%)	22 (24%)	12 (13%)	6 (6%)	18 (20%)	1 (1%)	9 (10%)
Moderately differentiated	60 (66%)	17 (18%)	15 (16%)	67 (73%)	37 (40%)	12 (13%)	56 (62%)	6 (6%)	17 (18%)
Poorly differentiated	0 (0%)	0 (0%)	0 (0%)	1 (1%)	1 (1%)	1 (1%)	0 (0%)	0 (0%)	0 (0%)
Recurrence	$p = 0.043$								
No	46 (50%)	11 (11%)	14 (15%)	48 (51%)	22 (23%)	11 (11%)	39 (42%)	45 (48%)	13 (14%)
Yes	39 (42%)	12 (13%)	9 (9%)	44 (47%)	28 (30%)	8 (8%)	37 (40%)	40 (43%)	13 (14%)
Tumor location									
Right colon	29 (31%)	10 (10%)	8 (8%)	34 (36%)	17 (18%)	6 (6%)	27 (29%)	3 (3%)	10 (10%)
Transverse colon	7 (7%)	1 (1%)	0 (0%)	6 (36%)	4 (4%)	0 (0%)	5 (5%)	1 (1%)	2 (2%)
Left colon	48 (52%)	11 (11%)	15 (16%)	51 (54%)	29 (31%)	13 (13%)	43 (46%)	3 (3%)	14 (15%)
Right and left Colon	1 (1%)	1 (1%)	0 (0%)	1 (1%)	0 (0%)	0 (0%)	1 (1%)	0 (0%)	0 (0%)

TC: tumor cells, CAF: Cancer Associated Fibroblasts, MIC: mononuclear inflammatory cells, TLR toll-like receptor, MMP: metalloprotease

grade or tumor location; however, the global expression of TLR4 was associated with age ($p = 0.010$) and tumor size ($p = 0.004$) (Table 2).

When we consider the expression of these factors by each cell type (tumor cells, CAFs, and MICs), our results show that TLR4 expression by tumor cells was associated with recurrence (Table 3). But, no significant associations between clinicopathological characteristics and TLR4, MMP11 and MMP13 cell type expressions were found.

TLR4, MMP11 and MMP13 Expression and Prognosis

The potential relationship between TLR4, MMP11 and MMP13 expression and prognostic variables (global recurrence, local recurrence, carcinomatosis, distant metastasis and overall survival) were evaluated in the 96 patients included in this study. Our results did not show significant association between global expression (score values) of each factor and prognostic variables (data not shown). However, TLR4 expression by tumor cells was significantly associated with a lower rate of global tumor recurrence ($p = 0.024$), a lower rate of carcinomatosis development ($p = 0.001$) (Fig. 2), a lower rate of distant metastasis development ($p = 0.008$) and longer overall survival ($p = 0.001$). This factor identifies 92.4% of patients with having a low risk of tumor recurrence of CRC. On the other hand, MMP11 expression by fibroblasts was significantly associated with a high rate of distant metastasis development ($p = 0.034$), concurrence of hepatic metastasis ($p = 0.029$) and shorter overall survival ($p = 0.035$) (Fig. 3), identifying therefore a 53.8% of patients with having a high risk of tumor recurrence and death by tumor progression.

To improve the prognostic value of the MMP11 expression by fibroblasts, we analyzed its combination with clinicopathological parameters, such as patient's age, sex, tumor size, histological grade and tumor localization. Our results indicate that the concurrence of factors such as older age (≥75 years) and the expression of MMP11 by fibroblasts was highly associated with a shortened relapse-free survival ($p = 0.025$) in our patient's population (Fig. 4). However, we found no significant association with overall survival (data not shown).

With regard to the MMP13 expression by the different cell types, we found no significant associations with prognosis (data not shown).

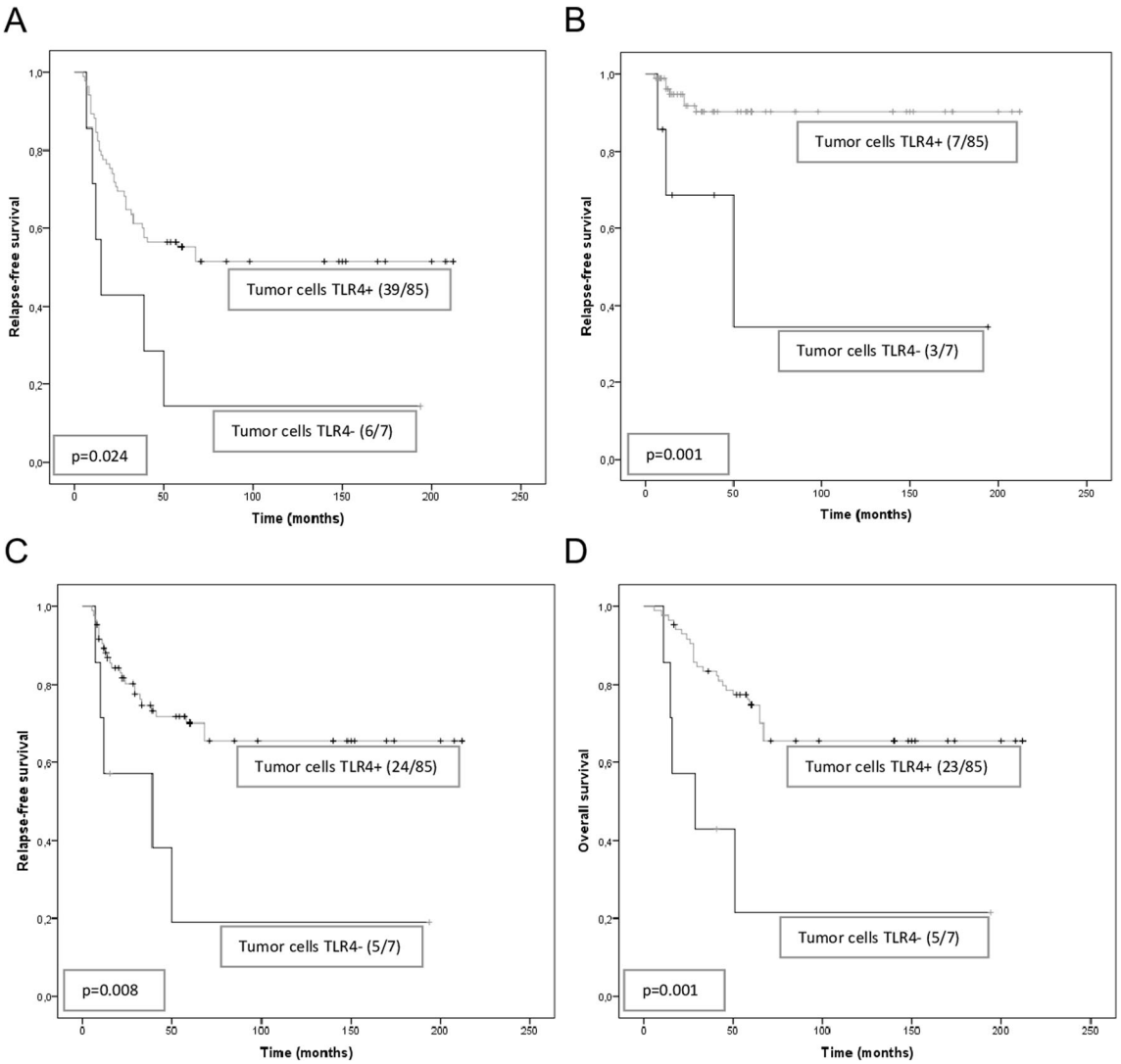

Fig. 2 Prognostic significance of expression of Toll-like receptor 4 (TLR4) in colorectal carcinomas. Kaplan–Meier survival curves for relapse-free survival and overall survival, as a function of TLR4 expression by tumor cells. TLR4 expression association with: **a** global tumor recurrence, **b** carcinomatosis development, **c** distant metastasis development, and **d** overall survival. The ratio of number of events/total cases is indicated in each graph

Discussion

Our results reveal a variability of the analyzed factors among stage II CRCs, which seems to correspond to the biological heterogeneity of these tumors. We not found significant associations between the global expression (score values) of all of these factors in our CRC population and clinicopathological parameters or prognostic variables. However, our results show significant relationships between either TLR4 expression by tumor cells and MMP11 expression by stromal fibroblasts with prognostic variables, which seems to demonstrate the importance of the cellular type expressing each factor in the tumoral context.

Similarly to our prior report in a global population of patients with CRC [22], our results reveal that TLR4 expression by tumor cells is associated with a good prognosis in stage II CRCs. Supporting this finding, it has been reported that tumor cells can be induced, through the TLR4 pathway, to produce IFNβ and positively contribute to antitumor immune response [47]. However, the finding of TLR4 non-expression by tumor cells identifies only a 7.6% of tumors with high rate of tumor recurrence in patients with stage II CRC, which limit its clinical prognostic value. It was of note to said that TLR4 expression by CAFs has been associated with a high rate of tumor recurrence in an overall population of patients with CRC [22], but this not occur in stage II CRC. This unfavorable influence of TLR4 expression by CAFs on the course of the disease may be based on the immunologic response generated by cancer cells, to induce matrix metalloproteases production that allows the invasion and metastasis in CRC [41]. Indeed, the molecular pathway that links inflammation to the acquisition of metastatic capacity during tumor progression involved the regulation of matrix metalloproteases by TLRs [48–52].

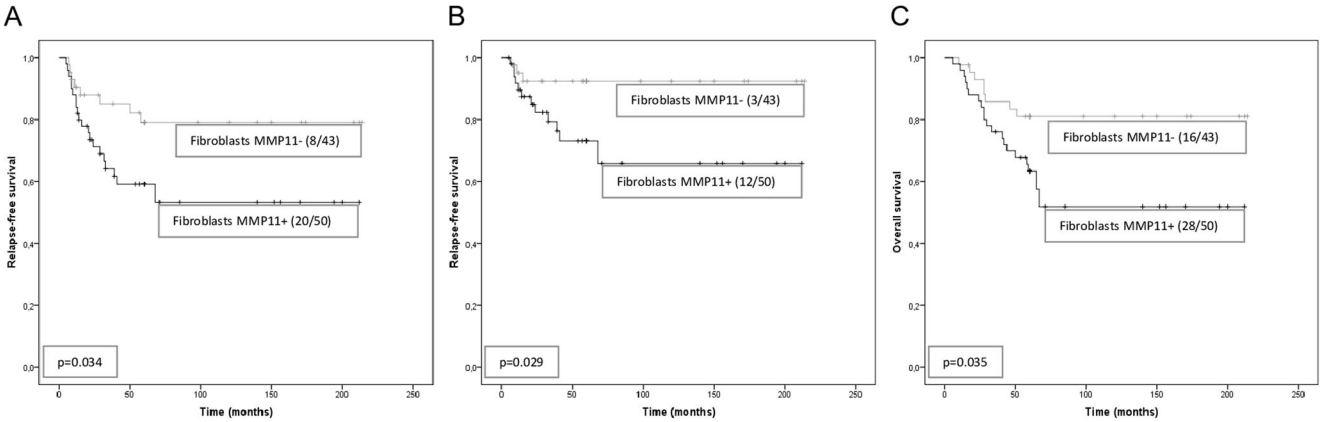

Fig. 3 Prognostic significance of stromal expression of Matrix Metalloprotease 11 (MMP11) in colorectal carcinomas. Kaplan–Meier survival curves for relapse-free survival and overall survival, as a function of MMP11 expression by stromal fibroblasts. MMP11 expression association with: **a** distant metastasis development, **b** concurrence of hepatic metastasis, and **c** overall survival. The ratio of number of events/total cases is indicated in each graph

MMP11 (also named Stromelysin 3) is normally involved in extracellular matrix degradation, tissue remodeling and wound healing. However, several studies have shown that MMP11 expression has been associated with poor prognosis in several carcinomas [43, 53–55]. In our study on stage II CRC, as in another study on a wide CRC population [22], we found that MMP11 expression by CAFs is associated with a poor prognosis and a shortened survival, allowing the identification of 53.8% of patients with a high risk of tumor recurrence and death due to tumor progression. In addition, MMP11 expression by CAFs was associated with the development of hepatic metastasis in stage II CRC patients. These findings reaffirm that tumor/stroma interactions play a very important role in tumor progression [56, 57]. There is also evidence suggesting that the expression of MMP11 by CAFs could be used as a new potential target for the development of vaccines and anti-tumor immune therapy [58].

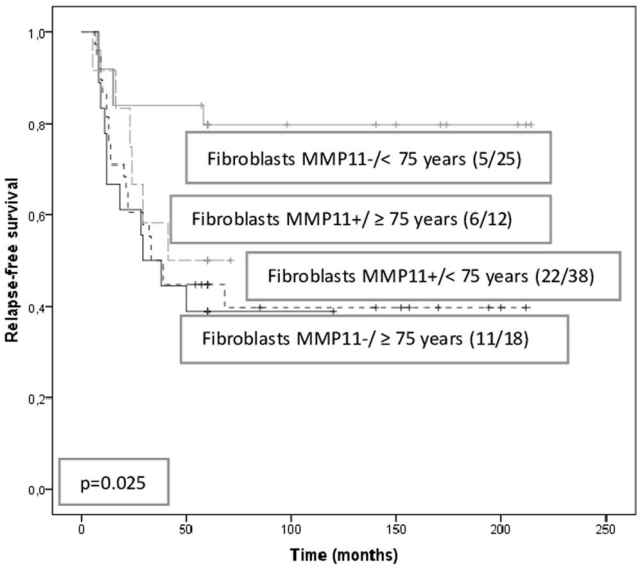

Fig. 4 Prognostic significance of stromal expression of Matrix Metalloprotease 11 (MMP11) and older age in colorectal carcinomas. Kaplan–Meier survival curve for relapse-free survival as a function of MMP11 expression by stromal fibroblasts and older age (≥75 years). MMP11 expression and older age association with distant metastasis development. The ratio of number of events/total cases is indicated in the graph

Since the expression of MMP13 has not been significantly associated with clinicopathological characteristics or prognosis of patients, and in order to improve the prognostic prediction of the MMP11, we analyzed the relevance of the combination of factors. We found that patients having <75 years and without MMP11 expression by CAFs have a specially good prognosis. This finding seems relevant given that advanced age (≥ 75 years) at diagnosis is a recognized predictor of poor outcomes in CRC in general [59], and in stage II CRC in particular [60]. The negative influence of an advanced age on cancer-specific outcome could be related to increased levels of comorbidity, frailty, and chronic systemic inflammation among the elderly [59, 61]. Interestingly, these factors in turn may also lead to an augmented inflammatory response which is associated with poorer cancer-specific survival [62], including patients with stage II CRC [63].

In summary, our results led us to consider that the analysis of the expression of TLR4 by tumor cells and MMP11 by fibroblasts could be very useful in patients with stage II CRC, in order to improve their prognostic evaluation.

Author Contributions NE, JFC and FV designed the study, analyzed and interpreted data. NE, JFC, SC, LOG, JLGM and AA collected data, carried out experiments, analyzed data and generated the figures. All authors were involved in writing the paper and had final approval of the submitted and published version. FV accepts full responsibility for the work and/or the conduct of the study, had access to the data, and oversaw the decision to publish.

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Compliance with Ethical Standards

Ethical Approval The study is adhered to National regulations, and was approved by the Fundación Hospital de Jove Ethics and Investigation Committee.

Conflict of Interest The authors declare that they have no conflicts of interest.

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