



Matrix metalloproteinase 9 -1562C/T polymorphism increased protein levels in patients with colorectal cancer in a sample from southeastern Brazil

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ABSTRACT. We examined whether the allelic and/or genotypic profile of locus -1562C/T of the matrix metalloproteinase (MMP-9) gene influences the protein expression levels of MMP-9 in patients with colorectal cancer (CRC) compared with controls. A total of 104 patients with CRC and 84 controls were evaluated. Peripheral blood was collected from both groups and DNA extraction was performed for -1562C/T genotyping; the plasma was used for MMP-9 quantification. The CT genotype was associated with increased MMP-9 expression ($P = 0.0211$). High levels of protein, independently of polymorphisms, were observed in the patient group ($P < 0.0001$) compared to controls. Mucinous tumors with signet ring cells were more frequent in females ($P = 0.0177$). Overall, patients older than 50 years showed a significant risk of developing CRC ($P = 0.0001$). MMP-

9 plasma expression was increased in patients with CRC compared to controls, particularly in those with the heterozygous -1562C/T genotype.

Key words: Colorectal cancer; Matrix metalloproteinase-9; Polymorphism -1562C/T

INTRODUCTION

Colorectal cancer (CRC) is considered to be a disease of aging (Farhoud et al., 2002), with a high incidence in individuals over 50 years of age (Saeed et al., 2013). The disease is associated with several risk factors, including ethnicity, age, lifestyle, diet, family history, smoking, alcohol, obesity, depression, and environmental factors (Cruz et al., 2004; INCA, 2012). Colorectal cancer is the third most common type of cancer in men and second in women worldwide. In southeastern Brazil, CRC is the second most common type of cancer in both men and women after non-melanoma skin cancer (INCA, 2014).

Colorectal adenocarcinomas (95-98% CRCs) can be characterized according to their production of mucus. Mucinous tumors are defined by the presence of extracellular mucin at more than 50% of the tumor volume and account for approximately 8-20% of all CRCs (Volpato and Koch, 2008; Rocha et al., 2010). The presence of signet ring cells is a variant of mucinous tumors and is found in 0.1-2.4% of CRCs. The presence of mucus in CRCs has been associated with more advanced stages of the disease and indicates a locally aggressive tumor with a poor prognosis (Volpato and Koch, 2008) compared to the non-mucinous type (Farhoud et al., 2002; Henrique-Filho et al., 2004). Life expectancy is less than 5 years in patients with mucinous CRC (Rocha et al., 2010).

Among the factors that can influence the clinical outcome of CRC patients, particularly regarding the development of invasive disease and metastasis, matrix metalloproteinase 9 (MMP-9) plays an important role. This gelatinase is released in response to a variety of stimuli, including the development of malignant cells (Nakahata et al., 2009; Zhang et al., 2012). In the promoter region of the MMP-9 gene, we found the single nucleotide polymorphism (SNP) C/T in locus -1562 with functional action. The presence of the T allele increased the levels of protein transcription, as it impairs the action of a transcriptional suppressor protein (Zhang et al., 1999; Langers et al., 2008). The association between MMP-9 expression and CRC has been described in several studies (Zuzga et al., 2008; Surlin et al., 2011). However, associations between the -1562C/T polymorphism and MMP-9 expression levels are still inconclusive (Peng et al., 2010; Liu et al., 2012; Herszényi et al., 2012; Zhang et al., 2012). Since no data are available regarding polymorphism and expression levels of MMP-9 in Brazilian CRC samples, we assessed this association in patients with CRC and to compare this information with clinical characteristics and controls.

MATERIAL AND METHODS

This study was approved by Santa Casa de Belo Horizonte Hospital Research Ethics Committee (CEP 22/2011). Blood samples were collected from 104 patients with CRC with a mean age of 61.7 ± 14.84 years (55 women and 49 men) followed at the Coloproctology Clinic of Santa Casa de Belo Horizonte Hospital and from 84 controls with a mean age of 38.7 ± 13.16 years (53 women and 31 men) recruited randomly at Santa Casa de Belo Horizonte Hospital among the patients' chaperons, excluding related individuals. Expression of MMP-9 was evaluated in 54

patients and 21 controls. Participants were age-matched to prevent bias. The mean age in the patient group was 60.2 ± 16.57 years (30 women and 24 men), with 52 ± 15.07 years (9 women and 12 men) in the control group. DNA was extracted from 500 μ L whole blood using the Genra Puregen Blood Kit (QIAGEN, Hilden, Germany) according to the manufacturer protocol. Genotyping of SNP -1562C/T (rs3918242) was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) as described by Palei et al. (2010). The expression of MMP-9 was assessed in plasma samples (patients and controls) using an enzyme-linked immunosorbent assay (ELISA) using the Human MMP-9 DuoSet Kit (R&D Systems, Inc., Minneapolis, MN, USA) following the manufacturer guidelines. The clinical data of patients included age, gender, presence of mucinous and signet rings cells, TNM status, and carcinoembryonic antigen (CEA) levels.

Statistical analysis

The GraphPad Prism 5.0 software was used for comparative analyses of polymorphism vs clinical variables (χ^2 test and Fisher exact test) and protein expression vs polymorphism and clinical variables (*t*-test or one-way ANOVA).

RESULTS

Expression and polymorphism of the MMP-9 gene

MMP-9 expression was significantly increased in CRC patients compared with controls (0.69 ± 0.08 vs 0.23 ± 0.04 ng/mL; $P < 0.0001$; Figure 1). When evaluating the polymorphism of the -1562C/T locus in relation to protein levels, we found significant differences only in the CRC group ($P = 0.021$), in which we observed higher MMP-9 expression in those harboring the heterozygous CT genotype (1.0 ± 0.2 CT vs 0.6 ± 0.1 ng/mL CC; Figure 2). The same was not true for the control group (0.17 ± 0.04 CT vs 0.24 ± 0.05 ng/mL CC). All data are presented in Table 1. No associations were found between expression levels and/or polymorphism and other clinical data (tumor stage, metastasis, and mucinous status).

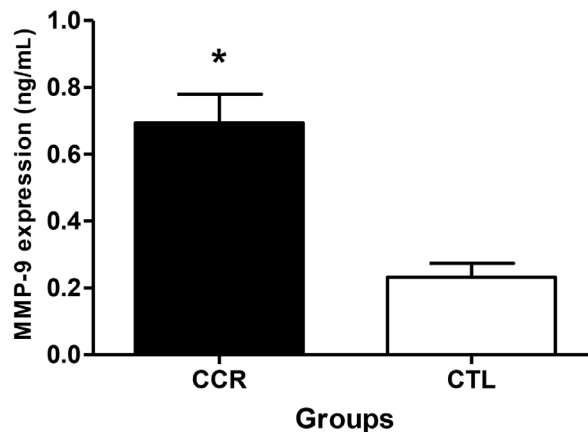


Figure 1. Mean MMP-9 expression in CRC and CTL groups. Box plot of means and standard errors (SE). * $P < 0.0001$.

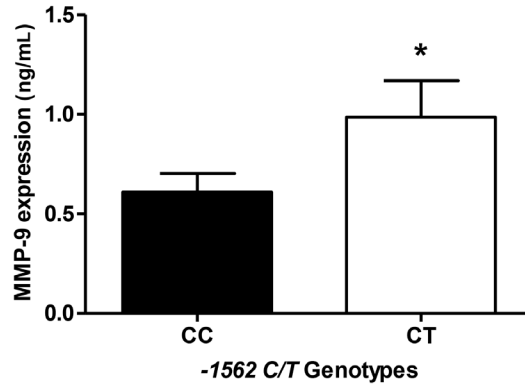


Figure 2. Mean genotypic expression of MMP-9 in the CRC group. Box plot of means and standard errors (SE). *P = 0.021.

Table 1. Comparison of MMP-9 protein expression levels between patients (CRC) and controls (CTL).

Characteristics		CRC (N = 54)	CTL (N = 21)	P
		Mean ± SE	Mean ± SE	
Protein expression (ng/mL)		0.69 ± 0.08	0.23 ± 0.04	<0.0001*
Polymorphism -1562 C/T in CRC	CC	0.6 ± 0.1	-	0.021*
	CT	1.0 ± 0.2	-	
Polymorphism -1562 C/T in CTL	CC	-	0.24 ± 0.05	0.779
	CT	-	0.17 ± 0.04	
	TT	-	0.173**	

C = cytosine; T = thymine; CC = Homozygous genotype C; TT = Homozygous genotype T; CT = heterozygous genotype; SE = standard errors; CRC = patients; CTL = controls; *P < 0.05, **N = 1.

Clinical features

In the analysis of clinical features, stratification of subjects by age (>50 years) revealed a strong association between CRC and age. Patients older than 50 years had an increased risk of CRC (OR = 17.26; 95%CI = 8.209-36.28; P < 0.0001; Table 2). Among the CRC patients, 31.7% (33/104) had tumors with a mucinous component. When the mucinous group was stratified into the presence or absence of signet ring cells, we found a significant difference for patient gender. In our samples, only 10 patients with mucinous tumors exhibited signet ring cells (10%), and 9 of these patients were women (90%) (OR = 9.39; 95%CI = 1.14-77.13; P = 0.0177; Table 3). Another significant association was found between CEA and metastatic tumors (MET) (P = 0.024), as these tumors showed an approximately 2-fold increase in CEA expression relative to non-metastatic tumors (15 ± 4.1 vs 7 ± 1.4 ng/mL).

Table 2. Clinical features of patients (CRC) and controls (CTL).

Characteristics	CRC		CTL		OR	95%CI	P
	N = 104	(%)	N = 84	(%)			
Age					17.26	8.209-36.28	0.0001*
<50 years	25	(24)	71	(84)			
>50 years	79	(76)	13	(16)			
Gender					0.6565	0.3649-1.181	0.1829
F	55	(53)	53	(63)			
M	49	(47)	31	(37)			
Skin color					-	-	0.1651
B	33	(32)	37	(44)			
P	53	(51)	32	(38)			
N	18	(17)	15	(18)			

OR = odds ratio; CI = confidence interval; F = female; M = male; B = white; P = brown; N = black; *P < 0.05.

Table 3. Occurrence of signet ring cells in relation to demographic variables.

Characteristics	Presence of signet ring cells		Absence of signet ring cells		OR	95%CI	P
	N = 10	(%)	N = 94	(%)			
Age					1.403	0.3341-5.888	0.7001
<50 years	3	(30)	22	(23)			
>50 years	7	(70)	72	(77)			
Gender					9.391	1.143-77.13	0.0177*
F	9	(90)	46	(49)			
M	1	(10)	48	(51)			

OR = odds ratio; CI = confidence interval; F = female; M = male; *P < 0.05.

DISCUSSION

MMP-9 expression in CRC

Increased levels of MMP-9 in the carcinogenic process have been described for various tumor types, including CRC (Felin et al., 2008). This enhanced expression is associated with angiogenesis, tumor progression, invasion, metastasis, and lower survival rates (Felin et al., 2008; Park et al., 2011; Herszényi et al., 2012; Zhang et al., 2012).

Some studies comparing the expression levels of the messenger RNA (mRNA) of MMP-1, -2, -3, -7, and -9 in the tissue from patients with CRC revealed increased expression in those MMPs, including MMP-9, compared to normal intestinal mucosa and/or adenomas (Luo et al., 2005). Other studies also found a significant increase in plasma MMP-9 levels in patients with CRC compared to controls (Wilson et al., 1999; Mroczko et al., 2010). Our results agree with those of previous studies, as we noted an approximately 3-fold increase in plasma MMP-9 levels in patients compared to controls, suggesting that the increase in MMP-9 was related to CRC in our samples. However, no significant association was found when we considered the metastatic cell profile and MMP-9 expression. These data corroborate those of Langers et al. (2012), Mook et al. (2004), and Felin et al. (2008), who also found no association between elevated MMP-9 expression and metastatic profile when analyzing tissue expression by immunohistochemistry (Mook et al., 2004; Felin et al., 2008; Langers et al., 2012). In contrast, Zhang et al. (2012), investigated MMP-9 immunohistochemistry expression in the tissues of patients and controls, observed significantly high MMP-9 levels in patients with metastasis, which was related to poor prognosis (Zhang et al., 2012). Similarly, Zeng et al. (1996) found that MMP-9 levels were 5-fold greater in CRC compared to the normal mucosa (Zeng et al., 1996). Other studies also found a similar relationship between MMP-9 and metastasis (Yamada et al., 2010; Chen et al., 2012; Herszényi et al., 2012). The contradictory results regarding MMP-9 expression and its relationship with invasion, lymph node involvement, and distant metastasis were because of the different sources of the samples.

MMP-9 gene polymorphism and CRC

The SNP identified in the promoter region of the MMP-9 gene, -1562C/T, has a functional allele-specific effect that plays a critical role in the regulation of gene transcription. This polymorphism is associated with the susceptibility to cancer, including CRC (Peng et al., 2010; Liu et al., 2012; Yang et al., 2012; Ji et al., 2013). The C allele is the most frequent in the world population, which was confirmed in our sample (data not shown); however, the T allele exerts a functional effect on protein synthesis of MMP-9 (Zhang et al., 1999; Langers et al., 2008). We found a positive relationship between the presence of the heterozygous genotype CT and an increase of approximately 1.5-

fold in the plasmatic expression of MMP-9, only for the CRC group. This enhanced expression in heterozygous individuals was likely related to the presence of the T allele, which, according to the literature, hinders the binding of a transcription repressor protein to induce increased expression of MMP-9 (Zhang et al., 1999; Langers et al., 2008). Our results corroborate those of Xing et al. (2007), who evaluated this polymorphism in CRC, and other studies investigating the same polymorphism in breast cancer (Chiranjeevi et al., 2014) and prostate cancer (Schveigert et al., 2013). However, when we took into account the -1562C/T polymorphism and the risk of CRC as well as the association of this polymorphism with other clinical and laboratory variables, we found no significant results. Similar findings were described by Langers et al. (2008), who found that high levels of MMP-9 were related to worse prognosis in patients with CRC, but found no association between the -1562C/T polymorphism and other clinical data.

Clinical features

Among the clinical and laboratory variables analyzed, three were significant: age, the mucus component with signet ring cells, and CEA levels.

When the groups were stratified by age, we found that patients older than 50 years were significantly more affected by CRC ($P = 0.0001$), confirming that age is a risk factor for the development of CRC, as demonstrated in several previous studies (Farhoud et al., 2002; Volpato and Koch, 2008; Park et al., 2011).

Adenocarcinoma with a mucinous component represents approximately 23% of tumors (Langers et al., 2008; Chen et al., 2012). According to the literature, this component, regardless of tumor differentiation status, implies a worse prognosis. Some reasons for the association of mucinous tumors with greater severity include local invasion causing the so-called “frozen pelvis” (Farhoud et al., 2002), peritoneal dissemination (Liu et al., 2012), and lymph node invasion (Henrique-Filho et al., 2004). The presence of signet ring cells is a variant of mucinous tumors occurring in approximately 0.1-2.4% of these tumors (Rocha et al., 2010; Hyingstrom et al., 2012; Nitsche et al., 2013). A German study examined the mucus component in patients with CRC tumors and found 11% (375 patients) of mucinous tumors and only 0.9% with signet ring cells, with a predominance of males in both cases (Nitsche et al., 2013). A study of North Americans showed similar frequencies: 10% of mucinous tumors, predominantly among males (54%), and only 1% of signet ring cells, with equivalence between genders (50%) (Hyingstrom et al., 2012). In our sample, the frequency rates of both mucinous tumors and signet ring cells were significantly higher than those described in the literature (32 and 10%, respectively). Interestingly, signet ring cells were present almost exclusively in females (90%; $P = 0.0177$). Although previous studies found no clear relationship between the presence of mucus and survival, most studies agree that these patients have worse prognosis and shorter survival compared to patients with non-mucinous tumors (Henrique-Filho et al., 2004; Chen et al., 2012; Numata et al., 2012).

The CEA is the most well-known of the tumor markers for gastrointestinal tract diseases, particularly CRC, and is used for the diagnosis, assessment, and as a predictor of disease recurrence (Li et al., 2009; Su et al., 2012). This marker is present in both embryonic and healthy adult tissue, but its concentrations in malignant tissues can reach up to 60-fold higher than the reference values (Boucher et al., 1989). In our samples, patients with metastatic CRC had plasma CEA levels that were at least 2-fold higher than those without metastasis. Our data corroborate the findings of Su et al. (2012), who evaluated CEA levels in patients with local recurrence, single,

and multiple metastases, with higher values for multiple metastases (Su et al., 2012). They also found a positive association between CEA levels and tumor size (Su et al., 2012), which was not observed in our sample. Svobodova et al. (2011) also found significantly high levels of CEA in patients with early-stage CRC and associated it with disease progression (Svobodova et al., 2011; Byström et al., 2012; Su et al., 2012). Another study reported a significant association between CEA expression and a mucinous component (Numata et al., 2012), which was not observed in our samples.

CONCLUSIONS

Plasma levels of MMP-9 were increased in our sample of southeastern Brazilian patients with CRC, which may have been induced by the heterozygous CT genotype of the MMP-9 gene, locus -1562. Women were more affected by mucinous CRC, particularly when signet ring cells were present.

Conflicts of interest

The authors declare no conflict of interest.

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