RESEARCH ARTICLE

Matrix Metalloproteinase-2 (-1306 C>T) Promoter Polymorphism and Risk of Colorectal Cancer in the Saudi Population

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Abstract

Background: Matrix metalloproteinase-2 (MMP-2) is an enzyme with proteolytic activity against matrix proteins, particularly basement membrane constituents. A single nucleotide polymorphism (SNP) at -1306, which disrupts a Sp1-type promoter site (CCACC box), results in strikingly lower promoter activity with the T allele. In the present study, we investigated whether this MMP-2 genetic polymorphism might be associated with susceptibility to colorectal cancer (CRC) in the Saudi population. We also analyzed MMP-2 gene expression level sin CRC patients and 4 different cancer cell lines. Materials and Methods: TaqMan allele discrimination assays and DNA sequencing techniques were used to investigate the $C^{-1306}T$ SNP in the MMP-2 gene of Saudi colorectal cancer patients and controls. The MMP-2 gene expression level was also determined in 12 colon cancer tissue samples collected from unrelated patients and histologically normal tissues distant from tumor margins. Results and Conclusions: The MMP-2 $C^{-1306}T$ SNP in the promoter region was associated with CRC in our Saudi population and the MMP-2 gene expression level was found to be 10 times higher in CRC patients. The MMP-2 $C^{-1306}T$ SNP is significantly associated with CRC in the Saudi population and this finding suggested that MMP-2 variants might help predict CRC progression risk among Saudis. We propose that analysis of this gene polymorphism could assist in identification of patient subgroups at risk of a poor disease outcome.

Keywords: Colorectal cancer - matrix metalloproteinases - single nucleotide polymorphism - ORs - odds ratios

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Introduction

Colorectal cancer (CRC) is one of the most frequent causes of cancer death in industrialized countries with a yearly incidence of 50 new cases for every 100,000 people in the population (Boyle and Ferlay, 2005). CRC is the third most common cancer in the world and its prevalence has been steadily increasing over the last century (Parkin et al., 2002; Hey et al., 2004). CRC is traditionally classified into sporadic and familial or hereditary forms and represents a complex disease which development is mediated by genetic and environmental factors (Potter, 1999; Hemminki and Czene, 2002). CRC is a disease of aging, more than 90% occur in persons 50 years or older, and the incidence is 50 times greater in 60-79 years old than people younger than 40 (Gail and Kevar, 2012). Genetic alterations in CRC are those that change the DNA sequence such as single nucleotide polymorphisms (SNPs), insertion-deletion mutations and rearrangements. These types of mutations typically alter the gene product by changing the amino acid sequence of protein or by altering the quantity of protein produced (Ajay and Boland, 2012). The matrix metalloproteinase, MMPs, constitute a family of secreted and membrane-associated zinc-dependent endopeptidases that are capable of selectively degrading a wide spectrum of extracellular matrix and non-matrix proteins (Simon et al., 2001). Currently upwards, the MMP family expanded to include 23 zinc-dependent endopeptidases, many of which were first identified by their over-expression in tumor cells (Laurie et al., 2012). They can be categorized by substrate specificity to give the collagenases, stromelysins, gelatinases and membrane

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type MMPs. The broad range of substrates conveys its pivotal role during both normal physiological processes such as embryonic development, bone remodeling, angiogenesis and nerve growth and pathological states as in arthritis, atherosclerosis, liver fibrosis and cancer (Woessner, 1998). The MMP-2 (gelatinase A) has type IV collagenolytic activity and is constitutively expressed by most connective tissues cells including endothelial cells, osteoblasts, fibroblasts and myoblasts (Strongin et al., 1995). Numerous investigators have demonstrated that MMP-2 is one of the essential players in promoting tumor invasiveness, and metastasis and the levels of MMP-2 expression can be correlated with tumor grade (Poulsom et al., 1992; Boag and Young, 1994). The MMP-2 gene is located on chromosome 16 at q13-21 and spans 27,049 bp with thirteen exons. There were 283 entries of SNPs for the MMP-2 gene in the public NCBI Single Nucleotide Polymorphism database (dbSNP;build 125:http://www.ncbi.nlm.nih.gov/SNP/) (Yihong et al., 2009). Functional SNP in the promoter region of the MMP-2 (the $C^{-1306}T$/rs 243865) which disrupts an Sp1-type promoter site (CCACC box) affect MMP-2 expression or activity and may predispose to disease conditions, especially in those individuals carrying the MMP-2 variants associated with increased MMP-2 concentrations (Flavia et al., 2013). In this study, we investigated whether this MMP-2 genetic polymorphism was associated with susceptibility to CRC in Saudi population. We also analyzed the MMP-2 expression in CRC tissues and control normal samples.

Materials and Methods

Samples collection

This study was conducted after review and approval of the Institutional Review Board of the Ethics Committee at King Khalid University Hospital in Riyadh, KSA. Blood samples were collected from 95 confirmed colon cancer patients (61 males and 34 females, age range, 26-80 years; mean age, 58.4 years) and 122 healthy controls matched for age and sex. Colon cancer tissue samples were also collected from 12 unrelated colorectal cancer patients 8 males and 4 females and histologically normal tissues in the distant margin to the tumor were collected at the time of endoscopy. The diagnosis of cancer was based on standard clinical, endoscopic, radiological, and histological criteria. Clinical and demographic characteristics were recorded, including age at diagnosis, gender, family history, smoking habits, disease behavior, disease location, and need for surgery.

Nucleic acids isolation and purification

Tissue samples to be used for RNA analysis were immediately submerged in RNAlater solution (Ambion, Courtabeuf, France) to avoid RNA degradation, stored at 4°C for 24h, and then stored at -20°C until needed. Genomic DNA was isolated from blood samples using QIAamp^{R} DNA Blood Min Kit Cat. No. 51106 (Qiagen). Samples of 30-60 mg of the preserved colorectal tissues were homogenized in RLT lyses buffer (Qiagen) supplemented with 1% 2-mercaptoethanol, using a rotor-stator homogenizer. Total RNA was extracted using the RNeasy Mini kit (Qiagen), with a DNA digestion step, according to the manufacturer's instructions. Elution was performed with 50μl nuclease-free water. Concentration, purity, and integrity of the isolated RNA were determined using the Agilent 2100 Bioanalyzer System and Agilent Small RNA analysis kit according to instruction provided by the manufacturer (Agilent Technologies, Waldbronn, Germany). Total RNA in aliquots of 1μg was retro-transcribed into single-stranded c-DNA using the ImProm-II Reverse Transcription System (A3800, Promega USA). Complementary DNA was synthesized by reverse transcription and used as a template for the quantification of MMP-2 gene expression level.

Cell lines and culture conditions

Colon cancer cell lines LoVo, HST-116 and SW480 were obtained from Dr Abdelillah Aboussekhra Research Laboratories, King Faisal hospital Riyadh, Saudi Arabia. Human lung adenocarcinoma epithelial cell line (A549) was obtained from ATCC (ATCC No.CCL-185^{TM}). These cell lines were cultured in Dulbecco's medium (DMEM) supplemented 100IU/mL of penicillin G and 10% fetal bovine serum. The medium was changed three times a week and when the culture reached 90% confluence, the cells were detached from the flasks using a 0.05% trypsin-0.1% ethylenediaminetetraacetic acid (EDTA) solution, washed twice and finally resuspended in DMEH-supplemented medium at a final concentration of 10^6 cells/mL to use for RNA isolation and cDNA synthesis.

Genotyping for MMP-2

Genotyping for the $C^{-1306}T$ (rs 243865) in the 5'-flanking region of MMP-2 gene was determined by real time polymerase chain reaction (RT-PCR) using TaqMan Allele Discrimination assay (Applied Biosystem, Carlsbad, CA, USA). Probes and primers used for the $C^{-1306}T$ genotyping assay were customized as follows: forward 5'-GCCATTGTCAATGTTCCCTAAAACA-3'; reverse 5'-TGACTTCTGAGCTGAGACCTGAA-3' and probes 5'-CAGCACTC[T/C]ACCTCT-3'. TaqMan PCR was performed in a total volume of 20μl containing 20ng of genomic DNA, 1× TaqMan master mix and 1× assay mix placed in 96-well PCR plate. Fluorescence from PCR amplification was detected using Chromo 4 detector (Applied Biosystem 7500 Fat Real Time PCR System) and analyzed with manufacturer's software.

PCR and sequencing

PCR was carried out in a final volume of 50 μl containing 25μl highfidelity PCR master mix (GE Healthcare, USA), 3μl of genomic DNA (50ng) and 3μl (30pmole) of each primer (MMP-2 forward 5'-TAGCTGTGATGATCAAGACATAATCG-3'; reverse 5'-AGACAGTTGAGAGATAAGCTGTGA-3'). The PCR condition was 1 cycle at 95°C for 5 min followed by 30 cycles at 95°C for 40 seconds, 54°C for 40 seconds, and 68°C for 1 min. The final extension step was carried out at 72°C for 5 min. The PCR products were analyzed using 2.5 % agarose gel stained with 0.5 μg/mL ethidium bromide and visualized by ultraviolet transilluminator. Sequencing of the PCR products was carried out according

to Sanger et al. (1977) using the MegaBACE 1000 DNA Sequencing System (Pharmacia/Amersham Co.). The chain termination sequencing reaction was conducted utilizing the DYEnamic ET terminator kit as an integral part of the MegaBACE 1000 DNA sequencing system. The sequencing reaction products were purified using DyeEx 2.0 Spin Kit (63206 Qiagen) and applied to MegaBace 1000 Sequencing machine.

Quantitative real-time RT-PCR

Quantitative PCR (qPCR) was carried out as previously described (Lionel et al., 2010), mRNA transcripts for MMP-2 was measured using the Applied Biosystem 7500 Fast real-time PCR detection system. Reactions were performed using a PCR SYBR Green supermix from Applied Biosystem. Primer used for MMP-2 was as follows; forward, 5'-ACCCATTTACACCTACACCAAG-3' and reverse, 5'-GTATACCGCATCAATCTTTTCCG-3'. GAPDH internal control primer was; forward, 5'-GGTATCGTCGAAGGACTCATGAC-3' and reverse, 5'- ATGCCAGTGAGCTTCCCGTTCAGC-3'. Primers were added to the reaction mix at a final concentration of 250nM. Five microlitres of each cDNA sample was added to a 20µl PCR mixture containing 12.5µl of SYBR Green supermix (Applied Biosystem), 0.5µl of each primers (MMP-2 or GAPDH) (eurofins MWG/Operon) and 7µl of RNase/DNase-free water. Each reaction was performed in a 7500 fast real time PCR Thermal Cycler. The thermocycling conditions were established as 5 min at 95°C, followed by 40 cycles of 15s at 95°C, 30s at 58°C, and 30s at 72°C, with each reaction done in triplicate. The specificity of each primer pair was verified by the presence of a single melting temperature peak. GAPDH produced uniform expression levels varying by less than 0.5 CTs between sample conditions and was therefore used as a reference gene for this study. The amplified products were run on an agarose gel to confirm that there were no spurious products amplified during the cycles. Results were analysed using the $2^{-\Delta\Delta Ct}$ (Livak) relative expression method.

Statistical analysis

The Fisher's exact test and the odd ratio (OD) with 95% confidence interval (CI) were calculated to test the association between cancer and the studied genetic polymorphisms and to describe the strength of the association. The associations were considered to be statistically significant if the Fisher's exact p value was less than 0.05 and if the 95%CI excluded the value 1.0. All statistical calculations were done using the MedCalc Software (Version 11.3.1.0© 2010 MedCalc Software bvba).

Results

The identification of MMP-2 $C^{-1306}T$ polymorphism was carried out by two different PCR based methods. The first method was the amplification of 490bp DNA fragment of MMP-2 promoter site (Figure 1 and 2) followed by sequencing strategy. The second method was the TaqMan Allele Discrimination Assay utilizing primers and probes that identify both C and T alleles.

A total of 95 CRC cases and 116 healthy controls were included in this study. Clinical characteristics of CRC cancer cases and healthy controls are given in Table 1. All the genotypic distributions were consistent with that expected in the Hardy-Weinberg model. Homozygous ancestral allele was used as a reference to determine the odds of acquiring CRC patients in relation to the other genotypes. The genotype distribution of the analyzed SNPs along with the corresponding odds ratio and significance are shown in Table 2. In the present study, we found a significant variation in the distribution of MMP-2 $C^{-1306}T$ genotypes between colorectal cancer cases and the matched healthy controls (p>0.05) as shown in Table 2. The frequency of MMP-2 $C^{-1306}T$ genotypes in colorectal cancer cases were 66 (0.70), 24 (0.25), and 5 (0.05) respectively, whereas as in healthy controls the frequencies were 92 (0.79), 23 (0.2), and 1 (0.01) respectively.

The homozygous variant $C^{-1306}T$ in colorectal cancer patients showed significant risk when compared to healthy individuals (OR: 6.970; CI: 0.796-61.054 and p-value 0.043) (Table 2). The frequency of MMP-2 $C^{-1306}T$ (T) variant genotype was higher in CRC cases (0.18) when

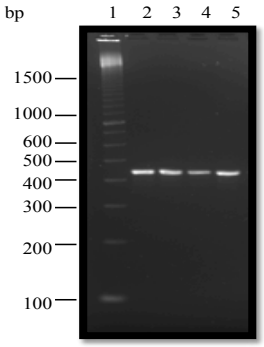

Figure 1. Agarose Gel (2.5%) Electrophoresis for PCR Products of MMP-2 Promoter (Lanes 2-5). Lane 1 represents 100 bp DNA molecular Weight markers

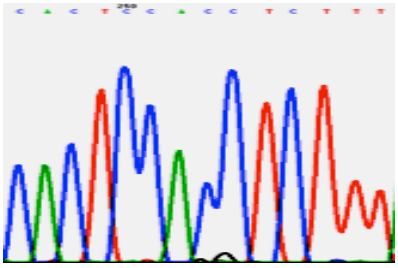

Figure 2. Sequence Analysis of MMP-2 Gene Promoter Shows Homozygous Genotype (C/C) and CCACC box

Table 1. Clinical Characteristics of Study Subjects

Variable		Colorectal cancer	Healthy controls	p value
Age group	≤60	50	67	0.45
	≥60	45	49	
Gender	Female	34	54	0.11
	Male	61	62	
Smoking status	Smoker	51	59	0.68
	Nonsmoker	44	57	
Tumor location	Colon	51		
	Rectum	39		

Table 2. Genotype Frequencies of MMP2 Gene Polymorphism in Colon Cancer Cases and Controls

MMP2 Genotype	Cases	Controls	OR	95%CI	X^2	p value
CC (wild)	66 (0.70)	92 (0.79)	Ref			
CT	24 (0.25)	23 (0.2)	1.455	0.76- 2.80	1.27	0.26
TT (variant)	5 (0.05)	1 (0.01)	6.97	0.80-61.05	4.07	0.04
CT+TT	29 (0.30)	24 (0.21)	1.684	0.90- 3.15	2.69	0.10
C	156 (0.82)	207 (0.89)	Ref			
T	34 (0.18)	25 (0.11)	1.805	1.03- 3.15	4.4	0.04

Table 3. Association Between MMP2 Gene Polymorphism and Clinicopathological Characteristics of Colon Cancer

Variables			Cases Group I	Controls Group II	OR	95% CI	X^2	p value
Age group:	≥60	CC	30 (0.67)	40 (0.82)	Ref			
		CT	11 (0.24)	9 (0.18)	1.63	0.60-4.43	0.92	0.34
		TT	4 (0.09)	0 (0)	11.95	0.62-230.4	4.97	0.03
		CT+TT	12 (0.33)	9 (0.18)	2.22	0.86-5.76	2.76	0.10
		C	71 (0.79)	89 (0.91)	Ref			
		T	19 (0.21)	9 (0.09)	2.65	1.13-6.21	5.27	0.02
	≤59	CC	36 (0.72)	52 (0.78)	Ref			
		CT	13 (0.26)	14 (0.21)	1.34	0.56-3.19	0.44	0.51
		TT	1 (0.02)	1 (0.01)	1.44	0.09-23.85	0.07	0.80
		CT+TT	15 (0.28)	15 (0.22)	1.35	0.58-3.13	0.48	0.49
		C	85 (0.85)	118 (0.88)	Ref			
		T	15 (0.15)	16 (0.12)	1.30	0.61-2.78	0.47	0.50
Gender Status:	Male	CC	41 (0.67)	54 (0.87)	Ref			
		CT	15 (0.25)	8 (0.13)	2.47	0.96-6.381	3.61	0.06
		TT	5 (0.08)	0 (0)	14.45	0.78-268.6	6.18	0.01
		CT+TT	20 (0.33)	8 (0.13)	3.29	1.32-8.22	6.91	0.01
		C	97 (0.80)	116 (0.94)	Ref			
		T	25 (0.20)	8 (0.06)	3.74	1.61-8.66	10.44	0.001
	Female	CC	25 (0.74)	38 (0.70)	Ref			
		CT	9 (0.26)	15 (0.28)	0.91	0.35-2.40	0.03	0.85
		TT	0 (0)	1 (0.02)	0.50	0.02-12.84	0.65	0.42
		CT+TT	9 (0.26)	16 (0.30)	0.86	0.33-2.23	0.1	0.75
		C	59 (0.87)	91 (0.84)	Ref			
		T	9 (0.13)	17 (0.16)	0.82	0.34-1.95	0.21	0.65

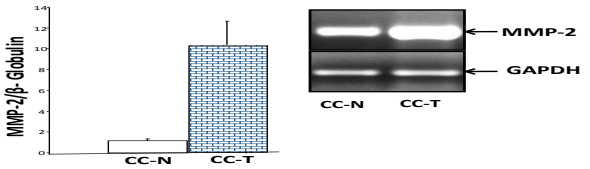

Figure 3. Expression of MMP-2 Gene in Colon Cancer Tissues (CC-T) and Normal Tissues (CC-N) Relative to GAPDH Gene

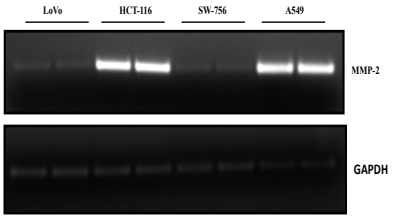

Figure 4. Expression of MMP-2 in Different Cancer Cell Lines as Compared to GAPDH

compared to healthy controls (0.11) (OR, 1.805; CI: 1.034-3.149 and p value 0.035) (Table 2).

In Saudi CRC population, the median age of onset of colorectal cancer is 60 years (Saudi Cancer Registry., 2007). To evaluate the association of MMP-2 $C^{-1306}T$ with the age at diagnosis of colorectal cancer, we stratified the CRC cases as ≤59 (n=50) and >60 (n=45) years of age and these patient groups were compared with the respective control age groups. Interestingly, both the homozygous 'TT' (OR: 11.951; CI: 0.620-230.4; p=0.02572) and T allele (OR: 2.646; CI: 1.129-6.205; p=0.02175) were significantly associated with higher risk of acquiring colorectal cancerogenesis in older aged patients (Table 3).

We conducted the association of CRC risk with the individual SNPs based on the gender of the patient. The genotype distribution in the male (n=61) and female (n=34) CRC patient groups were compared with the respective male and female control subjects. Interestingly, both the homozygous 'TT' (OR: 14.446; CI: 0.777-268.6; p=0.01293) and T allele (OR: 3.737; CI: 1.612-8.661; p=0.00124) were significantly associated with higher risk of acquiring colorectal cancerogenesis in males (Table 3).

The expression level of MMP-2 gene was 10 times higher in CRC samples as compared with control samples (Figure. 3). Colorectal cancer cell lines LoVo, HST-116, SW-480 and Human lung adenocarcinoma epithelial cell line, A549 showed CC genotype. The MMP-2 expression in HST-116 and A549 were much higher than that of LoVo and SW-480 cell lines (Figure. 4).

Discussion

The human matrix metalloproteinase-2 possesses proteolytic activity against type IV collagen, a major constituent of the basement membrane, and is therefore implicated in an extensive array of pathologies including atherogenesis, arthritis and tumor growth and metastasis

(Clifford et al., 1984; Itoh et al., 1998; Yrjo et al., 1999; Fang et al., 2000). Thus, any naturally occurring genetic variants that directly affect gene expression, and/or protein function would be expected to impact on progression of pathological processes involving tissue remodeling. Recently, there are a number of function altering polymorphisms found in the promoter of MMP-2. Some of these polymorphisms have allele-specific effects on the regulation of MMP-2 gene transcription and are associated with the development and progression of several diseases including CRC (Jormsjo et al., 2000; Enaping et al., 2004; Priyanka et al., 2012). In the present study, we investigated the role of MMP-2 $C^{-1306}T$ polymorphism within the promoter site in CRC in the Saudi population. Our results demonstrated a significant difference in MMP-2 allelic variant distribution with a 1.909 exceeding risk of colorectal cancer for C/C and a 7.424 for T/T homozygous patients (Table 1) and this polymorphism may influence the ability in CRC invasion. This molecular epidemiological results are consistent with previous findings of Grieu et al. showing that, the C>T transition at -1306, which disrupt the Sp1-type promoter site and results in a strikingly lower promoter activity with the T allele of the MMP-2 gene (Grieu et al., 2004). A study in Chinese population showed that the -1306 CC genotype to double breast cancer probability (Zhou et al., 2004). Contrarily, Roehe et al. (2007) reported that, no association between -1306 C>T polymorphism and breast cancer. Studies in a Swedish population reported that, no association was found between the MMP-2 promoter polymorphisms and CRC (Elander et al., 2006). Enping et al. (2004) investigated the role of -1306 C>T polymorphism within the MMP-2 gene promoter in CRC in the Chinese population and they demonstrated a significant difference in MMP-2 allelic variant distribution for C/C homozygous patients. Our study showed that, a higher CRC risk in men with than in women with the same genotype (41 versus 25 cases) as shown in Table 2. This was in contradictory with the finding of Enpling et al., who observed that a higher CRC risk was in women with the C/C genotype than in men and they suggested that a gender-specific effect to the MMP-2 polymorphism. One possible explanation was introduced by Singer et al. (1997) is that, the activity of MMP-2 may be regulated by sex hormones since the secretion and activation of several MMPs including MMP-2 were inhibited by progesterone which suggests that the mean level of MMP-2 may be less in women than in men during their lifetime (Singer et al., 1997).

The association between the MMP-2 polymorphism and an invasive phenotype in CRC is consistent with the biological function of MMP-2. High expression levels of MMP-2 in the malignant epithelium as well as in the surrounding stroma are associated with reduced survival of CRC patients (Collins et al., 2001). In the present study, MMP-2 mRNA was found to be up-regulated (10 times) in colorectal cancer tumor tissues compared to normal tissues (Figure 3). The MMP-2 promoter a 1.9 kbp in size and contains Sp1 a ubiquitously expressed transcription factor that binds to GC/GT-rich elements and regulates a variety of genes in a constitutive or inducible manner (Rutter et al., 1998; Simon et al., 2001). One of such motif, the sequence CCACC box, has been shown to be essential for Sp1 binding and promoter function in several genes by invariably activating transcription (Maouche et al., 1995; Alory et al., 1999). Sp1 is a multifunctional protein that can directly interact with the basal transcriptional complex as shown for the MMP-2 proximal promoter (Qin et al., 1999; Price et al., 2001), or alternatively function as a more general transcription factor and play an important role in directing tissue-specific expression (Block et al., 1996; Margana and Boggaram, 1997). Clearly, any variant that abolishes Sp1 binding, such as MMP-2 $C^{-1306}T$ polymorphism, has the potential to affect the level and specificity of gene transcription.

In conclusion, in summary this study indicated that the MMP-2 $C^{-1306}T$ polymorphism will be informative in tests of associations in a wide spectrum of pathologies in which a role for MMP-2 is implicated. In addition, this is the first study to demonstrate that MMP-2 $C^{-1306}T$ polymorphism may be associated with the risk of developing CRC in a Saudi population.

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