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The-1306C>T Polymorphism in the Promoter Region of *matrix metalloproteinase (MMP)-2* Gene in Colorectal Cancer Patients

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Abstract: The aim of this study was to investigate the relation between -1306C>T polymorphism in promoter region of the *MMP-2* gene and the risk of colorectal cancer (CRC) in Vietnamese patients. Tissue samples from 120 CRC patients and blood samples from 60 donors were analyzed in our study using polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) and DNA sequencing approach. We found that the incidence of -1306C>T was 13.6% and 21.7 % in the tissue of CRC patients and blood samples of controls, respectively. We further observed a tendency of higher risk of CRC in the patients harboring CC genotype. The CT genotype appeared more frequently in CRC patients with rectal tumor than that in the colon one ($P<0.05$). Moreover, the prevalence of CT genotype decreased when the size of tumor increased ($P<0.05$). There were no significant association between the given genotypes and the other pathological parameters (age, gender, differentiation, N, T and TNM stages) in the CRC patients. These findings implied the impact of -1306C>T polymorphism on transcriptional expression of *MMP-2* and proposed a potential approach for identification of population with high risk of CRC.

Keywords: -1306C>T polymorphism, colorectal cancer, MMP-2, PCR-RFLP.

1. Introduction

Colorectal cancer (CRC) is one of the three leading cause of death due to cancers worldwide [1]. It has been estimated that approximately 694.000 patients died from CRC in 2012 [2]. Recently, the incidence of CRC has gradually grown in many Asian countries including Vietnam. In 2012, CRC was the fifth common cancer in Vietnamese women [3]. Despite this worse incidence, national specific guidelines for CRC on the object of Vietnamese patients are still negligible.

Matrix metalloproteinases (MMPs), the members of calcium-dependent, zinc-containing endopeptidase family, are capable of degrading various components of the extracellular matrix (ECM) including collagens, gelatin, proteoglycan, elastins and matrix glycoproteins [4]. Hence, the elevated expression of MMPs has been showed to be related to multiple stages of cancer progression ranging from tumor invasion, metastasis, proliferation and adhesion [5].

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Functional polymorphisms (SNP) located in promoter region of *MMP-2* gene have been reported to contribute to oncogenesis and tumor progression by affecting the expression of the corresponding protein [6]. It occurs through modifying the binding sites of various transcriptional factors such as AP-2, p53, Sp1, and Sp3 [7]. The SNP C→T transition at -1306 position has been showed to disrupt CCACC box, Sp1-type promoter binding site, leading to the slip in the transcriptional activity, finally, the activity of MMP-2 [6]. The role of -1306C>T *MMP-2* polymorphism was proved in a wide range of cancers composing lung cancer [8], oral carcinogenesis [9], head and neck cancer [10]. However, these findings are not always in accordance with the others due to the typical characteristics of each population. In Vietnam, there is none preceding study concerning to this polymorphism in CRC. Therefore, we conducted this research in order to investigate the *MMP-2* -1306C>T polymorphism in a group of Vietnamese patients with CRC.

2. Material and Methods

Sample collection: One hundred and twenty pairs of tumor tissues and matching adjacent tissues from CRC patients were collected at K hospital and Viet-Duc hospital. Sixty blood samples from blood donors at National Hospital of Hematology and Blood Transfusion was used as the control group. All of the samples were snap-frozen and stored at -80^{o}C until DNA extraction.

DNA extraction: Genomic DNA was extracted from the samples using DNA purification kits (Thermo Scientific) according to the manufacturer's instructions. The concentration of extracted DNA was measured and then the total DNA collections were frozen at –20˚C for further analysis.

Primer design: The primers were designed utilizing Primer-BLAST (NCBI). The sequences of primers are forward primer: 5'- ACA AGT ATA TTG CTC CTG ATT CT - 3', reverse primer: 5'- GAC CTG AAG AGC TAA AGA GCT -3'.

PCR amplification: The PCR reaction was performed in a 12.5 µl volume with the following components: 6.25µl Maxima Hot Start PCR Master Mix 2x (Thermo Scientific); 0.25µl each primer 10µM; 2.5ng/µl template DNA in final concentration. The PCR reaction started with 5 min of incubation at 95^{o}C, followed by 35 cycles of 30s at 95^{o}C, 30s at 53^{o}C, and 30s at 72^{o}C, and a final elongation of 5 min at 72^{o}C. The PCR products were observed on the 1.7% agarose gel stained with ethidium bromide and visualized by ultraviolet transilluminator.

MMP2 genotyping: Genotyping was executed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) approach with *Bfa*I enzyme (FastDigest *Bfa*I (*Fsp*BI), Thermo Scientific) whose recognition site was 5'…C/TAG…3'. The 272 bp PCR fragment was digested with *Bfa*I and then separated by running on 10 % polyacrylamide gel stained with silver nitrate. The samples with homozygous CC represented two DNA bands at 184 bp and 88 bp, whereas, the homozygous TT was three DNA bands at 162 bp, 88 bp and 22 bp. The CT heterozygotes showed 4 bands at 184 bp, 162 bp, 88 bp and 22 bp. Finally, the PCR products were purified and sequenced according to Sanger's method.

Statistical Analysis: Association between *MMP-2* promoter polymorphism and pathological features of CRC patients was evaluated by Fisher exact test. Odds ratio (OR) and 95% confidence interval (CI) in addition to Chi-square test (χ^2) were utilized to compare the frequency of genotypes and alleles between CRC patients and healthy control group. In addition, 0.05 was considered as statistically significant standard for P-value.

3. Results and Discussion

3.1. MMP-2 –1306 C>T polymorphism

The existence of *MMP-2* -1306C>T was detected by PCR-RFLP approach. A 272 bp

DNA fragment of MMP-2 promoter was successfully amplified (Fig. 1A). The RFLP results of representative samples revealed the appearance of a band at approximatly 162 bp (lane 6- Fig. 1B), indicating the existence of -1306C>T polymorphism. A total of 16 out of 120 CRC cases (13.6 %) and 13 out of 60 samples from control group (21.7 %) showed the appearance of 3 bands: 184, 162 and 88 bp, implicating that those samples contained CT genotype (Fig. 1C).

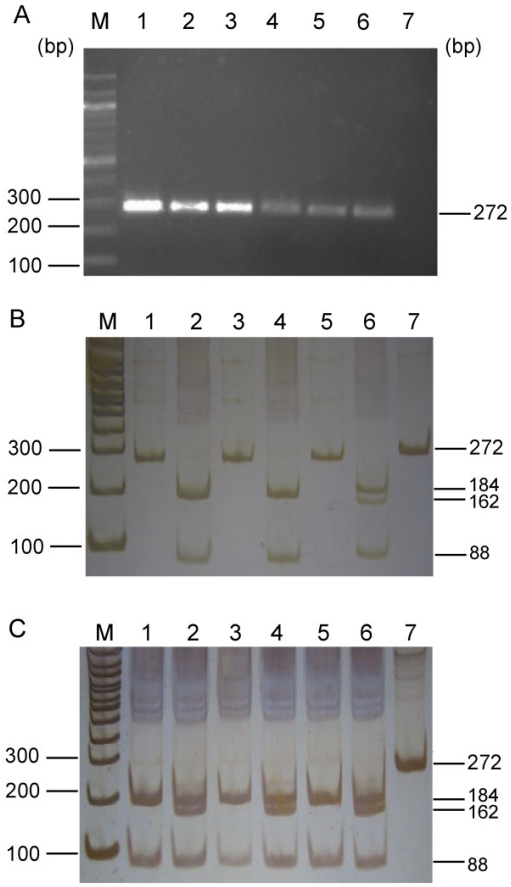

Figure 1. Electrophoresis images of PCR-RFLP analysis of *MMP-2* –1306 C>T polymorphism in CRC patients.

A) PCR products of *MMP-2* promoter (1.7% agarose gel, ethidium bromide staining); lane M: 100 bp DNA ladder; lanes 1, 2, 3: PCR products of tumor tissue, adjacent tissue and blood sample of patient #10370; lanes 4, 5, 6: PCR products of tumor tissue, adjacent tissue and blood sample of patient #3553; lane 7: negative control (PCR using water instead of total DNA)

B) PCR and restriction enzyme (RE) products of patient #16675 (10% polyacrylamide gel, silver staining); lane M: 100 bp DNA ladder; lanes 1, 3, 5: PCR products of tumor tissue, adjacent tissue, and blood sample; lanes 2, 4, 6: RE products of tumor tissue, adjacent tissue, and blood sample; lane 7: 272 bp PCR product as control (using water instead of enzyme).

C) RFLP analysis of -1306C>T polymorphism (10% polyacrylamide gel, silver staining); lane M: 100 bp DNA ladder; lanes 1, 3, 5: RE products of tumor tissue and adjacent tissue, and blood sample of patient #16678; lanes 2, 4: RE products of tumor tissue, adjacent tissue of patient #11777; lane 6: RE product of blood sample of patient #16689**;** lane 7: 272bp PCR product as control (using water instead of enzyme).

Next, the PCR products from promoter region of *MMP-2* gene of all tumor tissue samples containing CT genotype (found by PCR-RFLP) were sequenced to estimate the level of CT heterozygotes. The results represented diverse levels of CT heterozygotes across investigated samples (Fig. 2).

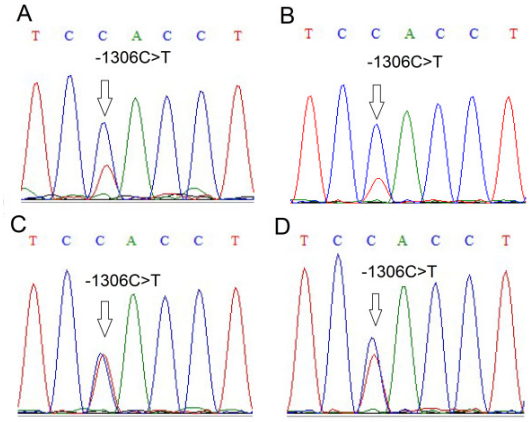

Figure 2. Sequence analysis of *MMP-2* gene promoter shows diverse levels of CT heterozygotes across investigated samples. A) patient #10370B, B) patient #42440B, C) patient #40683B, D) patient #8756B.

The frequencies of CC, CT and TT genotypes were 86.7 %, 13.6 % and 0 % in tumor samples, respectively (Table 1). It is interested that those values are totally equal with adjacent tissues. Hence, in further analysis, we focused on tumor tissues only. On the other

hand, in the control group, respective frequencies were 78.3 %, 21.7 % and 0 % for CC, CT and TT genotypes (Table 1). Moreover, we found that the difference in the genotype and allele frequency between the CRC and control group was not statistically significant (P>0.05). However, there was a tendency of the risk of CRC in the patients harbouring CC genotype (OR = 1.798; 95 % CI = 0.801-4.037) as well as C allele (OR = 1.701; 95 % CI = 0.79-3.664) (Table 1).

Table 1. Genotype and allele frequencies of MMP-2 polymorphism in CRC patients and controls

	Cases (n = 120) % (n)	Control (n = 60) % (n)	P	OR (95% CI)
Genotype				
CC	86.7 (104)	78.3 (47)	0.1517	1.798
CT + TT	13.6 (16)	21.7 (13)		(0.801-4.037)
Allele				
C	93.3 (224)	89.2 (107)	0.1709	1.701
T	6.7 (16)	10.8 (13)		(0.79-3.664)
OR: Odds ratio of CC genotype relative to CT genotype, 95 % CI: 95 % Confidence interval				

3.2. Association between the genotypes of MMP-2 -1306C>T and pathological features of CRC patients

The association between genotype distribution of *MMP-2* and pathological features was presented in Table 2. The result revealed that the frequency of CT genotype was remarkable higher in CRC patients with rectal tumor than that with the colon one (P<0.05). Moreover, the appearance of CT genotype was less frequent (P<0.05) when the tumor size increased. For the remaining features, no evidence of the association was detected.

Table 2. Association between the genotypes of MMP-2 -1306C>T and pathological features of CRC patients

Features	**Number**	**Genotype % (n)**		**P**
		CC	**CT+TT**	
Age				0.3708
<50	81	88.9 (72)	11.1 (9)	
≥50	34	82.3 (28)	17.7 (6)	
Gender				0.6033
Male	57	87.7 (50)	12.3 (7)	
Female	60	83.3 (50)	16.7 (10)	
Tumor location				0.0005
Colon	47	100 (47)	0 (0)	
Rectal	70	73.8 (54)	26.2 (16)	
Differentiation				0.8298
Poor	9	100 (9)	0 (0)	
Moderate	46	87.0 (40)	13.0 (6)	
High	12	91.7 (11)	8.3 (1)	
Tumor size (cm)				0.0057
<3.0	43	79.1 (34)	20.9 (9)	
≥3; ≤3.5	41	82.9 (34)	17.1 (7)	
>3.5	35	100 (35)	0 (0)	
N stages				0.4055
N_0	73	83.6 (61)	16.4 (12)	
$N_{1,2}$	42	90.5 (38)	9.5 (4)	

T stages				0.0651
$T_{1,2}N_-M_-$	61	80.3 (49)	19.7 (12)	
$T_{3,4}N_-M_-$	54	92.6 (50)	7.4 (4)	
TNMclassification*				0.4055
Stage I, II	73	83.6 (61)	16.4 (12)	
Stage III, IV	42	90.5 (38)	9.5 (4)	

*Stage I: $T_{1-2}N_0M_0$, II: $T_{3-4}N_0M_0$, III: $T_-N_{1-2}M_0$, IV: $T_-N_-M_1$

Our study demonstrates that the proportion of -1306C>T polymorphism on *MMP-2* promoter was higher in the control group than that in the patient group. Despite of the argument with regard to the association of any *MMP* gene polymorphism with CRC progression, our result is consistent to several previous reports. Kang et al. (2011) showed the predominance of CC genotype compared to CT and TT in both the CRC patient and control groups in Korean CRC patients [11]. Reversely, Shalaby et al. (2014) confirmed that -1306C>T polymorphism was more common in colon cancer patients than that in the control in Saudi population [12].

The promoter region of *MMP-2* gene contains a wide range of binding site for multiple transcription factors such as Sp1, Sp3, AP-2 and p53. Polymorphism at the specific position -1306 is supposed to disturb the binding affinity of transcription factors, resulting in the lower promoter activity. Price et al. (2001) showed a remarkable lower promoter activity of *MMP-2* due to the -1306C>T polymorphism that occured in the CCACC box of the Sp1 binding site and eliminated transcriptional effect [6].

In terms of pathological features, no association between the genotype distribution of -1306C>T and given pathological characteristics (age, gender, differentiation, N, T and TNM stages) was observed ($p>0.05$, Table 2). Similarly, Elander *et al.* (2006) showed no significant relationship between the *MMP-2* -1306 C>T polymorphism and clinicopatholgical parameters or susceptibility of CRC in Sweden patients [13]. On the contrary, Xu *et al.* (2004) found the evidence that individuals with CC genotypes faced with higher risk (OR=1.959, 95% CI=1.06-3.64) of developing CRC compared with those harbouring CT or TT genotypes in Chinese CRC patients [14]. Moreover, the serosa/adventitia layer involvement was more common in CRC patients with CC genotype than CT+TT. Hence, the authors concluded that *MMP-2* -1306 C>T polymorphism may contribute to the CRC development and invasion in the Chinese population.

Our result showed the higher frequency of the *MMP-2* –1306 C>T polymorphism in patients with smaller tumor size. This observation can be attributed to the requirement of MMP-2 expression in the progression of CRC tumor. The higher level of MMP-2 activity associated with larger tumor size, leading to destroy the surrounding connective tissues and invase [15]. Consequently, the fraction of –1306 C>T polymorphism reducing expression of MMP-2 will be inhibited.

The correlation between the genotype of *MMP-2* –1306 C>T polymorphism and tumor location has not been reported so far. Xu et al (2004) did not find significant correlation between this polymorphism in promoter of *MMP-2* and the location of tumor [14]. Even though colon and rectal cancer share many features, a number of studies showed the difference in diverse molecular polymorphisms. For instance, comparing to rectal cancer, proximal colon cancer was more likely to have CpG island methylator phenotype and KRAS mutation [16]. It probably implies many undisposed characteristics and mechanism of colon and rectal cancer including the expression of MMP-2.

Moreover, due to the multiple functions of MMP-2 in various biological processes, the role

of the *MMP-2* –1306 C>T polymorphism seems to be complex and might differ across cancer types. Several studies showed the association with the CC genotype and lung cancer [17] as well as the TT genotype and breast cancer [18]. However, a dual effect of the TT genotype in breast cancer prognosis was showed to depend on the estrogen receptor status [18].

In this study, we found the difference in the genotypic distribution between tissue and blood samples taken from the same patient. Especially, in case of a patient whose blood sample gets CT genotype and tissue sample gets CC genotype, risk of cancer development is probably high (Figure 1B). From this result, we supposed that *MMP-2* -1306C>T polymorphism was a somatic mutation in Vietnamese patients with CRC.

Our results emphasize the potential application of *MMP-2* -1306C>T in the further understanding of CRC development and the necessary of investigation on larger sample size to profound molecular relation between -1306C>T and risk of CRC.

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Đa hình -1306C>T trong vùng promoter của gen *MMP-2* ở bệnh nhân ung thư đại trực tràng

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Tóm tắt: Nghiên cứu được thực hiện nhằm khảo sát mối liên quan giữa tần suất đa hình -1306C>T tại vùng promoter của gen *MMP-2* và nguy cơ mắc ung thư đại trực tràng (UTĐTT) trên đối tượng bệnh nhân Việt Nam. Mẫu mô từ 120 bệnh nhân ung thư đại trực tràng và mẫu máu từ 60 người bình thường được phân tích bằng kỹ thuật PCR-RFLP kết hợp giải trình tự ADN. Kết quả cho thấy đa hình -1306C>T xuất hiện với tần suất 13,6% ở bệnh nhân ung thư và 21,7% ở nhóm đối chứng. Hơn nữa, chúng tôi còn quan sát thấy rằng nguy cơ mắc UTĐTT có xu hướng cao hơn ở các bệnh nhân mang kiểu gen CC. Ở nhóm bệnh nhân ung thư trực tràng, kiểu gen CT xuất hiện với tần suất cao hơn ở nhóm bệnh nhân ung thư đại tràng (P<0,05). Bên cạnh đó, khối u có kích thước càng lớn thì tỷ lệ kiểu gene CT càng giảm (P<0,05). Tuy nhiên, không tìm thấy mối liên quan giữa kiểu gen và các đặc điểm bệnh học khác (tuổi, giới tính, độ biệt hóa, giai đoạn N, T và TNM) ở bệnh nhân ung thư đại trực tràng. Kết quả nghiên cứu đã củng cố nhận định về vai trò của đa hình -1306C>T đối với hiệu quả phiên mã của gene *MMP-2* và khả năng ứng dụng nó như một một công cụ tiềm năng trong xác định nhóm các bệnh nhân có nguy cơ cao mắc ung thư đại trực tràng.

Từ khóa: Đa hình -1306C>T, MMP-2, PCR-RFLP, ung thư đại trực tràng.