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석사학위청구논문

Association between Tissue Inhibitor of Metalloproteinase (TIMP)-2, Matrix Metalloproteinase (MMP)-2 and MMP-9 Polymorphisms and Colorectal Cancer

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김선정의 석사학위 논문으로 인준함.

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논 문 개 요

Tissue inhibitor of metalloproteinase-2(TIMP-2)는 조직, 혈관, 임파조직에서 MMP-2 효소의 기능을 억제시키고, Metalloproteinase-2 (MMP-2)와 MMP-9 은 기저막을 구성하는 Type Ⅳ collagen 과 gelatine 을 분해하여 암세포의 침윤과 전이를 가능하게 한다. 또한 TIMP-2, MMP-2, MMP-9 는 대장암에서 과발현되어 전이와 침윤, 증식, 억제와 관련이 있다고 보고되고 있다. 이 논문에서는 한국인 대장암 환자 347 명, 염증성 대장질환(IBD) 환자 33 명과 건강인 312 명을 대상으로 *TIMP-2, MMP-2, MMP-9* 유전자의 단일염기다형성과 일배체형을 대장암의 발병 연령, 림프절의 전이 여부, 조직학적 분화도, 혈청 CEA 의 임상적 특징과 연관하여 분석하였다.

*TIMP-2 -418*G/*G, 303*G/*G (Ser101Ser)*의 동종접합자의 빈도와 *TIMP-2 -418G-303G* 의 일배체형 빈도는 대장암 환자군에서 건강인보다 통계적으로 유의하게 높았다(p = 0.024, OR = 1.4, 95% CI = 1.05 - 1.98; p <.0001, OR = 4.0, 95% CI = 2.67 - 6.02; p <.0001, OR

= 2.0, 95% CI = 1.55 - 2.51). 또한 *MMP-9 -1562*C/*C* 의 동종접합자의 빈도는 대장암 환자군에서 건강인보다 통계적으로 유의하게 높았다(p = 0.026, OR = 1.5, 95% CI = 1.05 - 2.21). 반면에, *MMP-2 -1575*G/*G* 의 동종접합자의 빈도와 *MMP-2 -735C-1575G* 의 일배체형 빈도는 대장암 환자군에서 건강인보다 통계적으로 유의하게 낮았다(p = 0.007, OR = 0.6, 95% CI = 0.42 - 0.87; p = 0.002, OR = 0.7, 95% CI = 0.56 - 0.88). 그 외 *MMP-2 -735C>T* 의 단일염기다형성은 대장암 환자군과 건강인에서 그 빈도의 차이가 없었다. 통계적으로 유의한 결과를 보인 *TIMP-2 -418G>C*, *TIMP-2 303G>A*, *MMP-2 -1575G>A*, *MMP-9 -1562C>T* 의 단일염기다형성과 대장암 환자에서의 임상적 특징과는 통계적으로 유의한 차는 없었다. 또한 *TIMP-2 -418G>C*, *TIMP-2 303G>A*, *MMP-2 -735C>T, MMP-2 -1575G>A*, *MMP-9 -1562C>T* 의 단일염기다형성은 IBD 환자군과 건강인에서 그 빈도의 차이가 없었다.

결론적으로, *TIMP-2 -418*G/*G*, *TIMP-2 303*G/*G (Ser101Ser)* 유전자형, *TIMP-2 -418G-303G* 일배체형과 *MMP-9 -1562*C/*C* 유전자형은 대장암에서 높은 감수성을 보이며 반면에, *MMP-2 -1575*G/*G* 유전자형과 *MMP-2 -735C-1575G* 일배체형은 대장암에서

낮은 감수성을 보인다. 또한 *TIMP-2 -418G>C, 303G>A, MMP-2 -1575G>A, MMP-9 -1562C>T* 은 대장암 환자군에서의 임상적 특징에 따른 차이가 없었다.

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Introduction

The tissue inhibitors of metalloproteinnases (TIMPs) are endogenous inhibitors of the matrix metalloproteinnases (MMPs). They have been shown to play important roles in multiple ways in all stages of cancer initiation and development. The balance between them is important in the maintenance of tissues and their disruption affects tissue homeostasis (Nagase et al., 2003; Ramnath et al., 2004; Zhou et al., 2004).

The TIMP family consists of four members: TIMP-1, -2, -3, -4. These molecules inhibit the proteolytic activity of activated MMPs by forming 1:1 stoichiometric inhibitory complex with the enzyme (Peres et al., 2005). In addition, numerous studies have indicated that TIMPs inhibit cellular invasion, tumorigenesis, metastasis and angiogenesis (Brew et al., 2000). There are at least 24 members of the MMP family that can degrade all constituents of connective tissue and thus facilitate invasion. MMPs can be grouped into collagenases (e.g. MMP-

1, -8, -13), gelatinases (e.g. MMP-2, -9), stromelysins (e.g. MMP-3, -10, -11) and matrilysins (e.g. MMP-7) according to their substrate specificity (Gentner et al., 2009). MMPs are capable of degrading both extracellular matrix (ECM) and basement membrane (BM), two physical barriers that play important roles in preventing expanding growth and migration of cancer cells. Therefore, overexpression of MMPs is associated with cancer cell invasion and metastasis (Zhou et al., 2004). MMPs also participate in the regulation of cellular processes such as differentiation, proliferation, angiogenesis and apoptosis by interacting with growth factors, cytokines, integrins and cell surface receptors (Gentner et al., 2009).

TIMP-2 functions to both inhibit MMP-2 activity and promote cell surface activation of pro-MMP-2 by MT-1-MMP through an MMP-dependent mechanism. And further TIMP-2 inhibits the growth of endothelial cells induced by basic fibroblast growth factor and regulates apoptosis through an MMP-independent mechanism (Stetler-Stevenson, 2008). So, TIMP-2 is not only a natural inhibitor of MMP-2 but a

suppressor of endothelial cell proliferation and angiogenesis as well. The complexity of TIMP-2 functions indicates a possibly multiple role in cancer progression and metastasis (Chen et al., 2007; Vairaktaris et al., 2007).

Tumor invasion and metastasis are considered to be the major causes in colorectal cancer. Recent researches in the field of mechanism for tumor invasion and metastasis have demonstrated that the degradation of ECM and BM is an essential step and the contribution of MMP-2 and MMP-9 is very important during this process. MMP-2 and MMP-9 are capable of degrading components of the BM and ECM. MMP-2 (Gelatinase A, 72 kDa type Ⅳcollagenase) primarily hydrolyzes gelatine and type Ⅳ collagenase, the major structural component of BM (Zhou et al., 2004). MMP-2 is most important in colorectal carcinogeneisis and promotes tumor invasion, metastasis. MMP-2 promoter has been showen to contain several cis-acting regulatory elements (Vasků et al., 2004; Wu et al., 2007). MMP-9 (Gelatinase B, 92 kDa type Ⅳ collagenase) cleaves type Ⅳ

collage and involves in cancer invasion and metastasis. The expression of MMP-9 is primarily regulated at the level of transcription (Ferrand et al., 2002). Furthermore, MMP-2 and MMP-9 appear to cooperate in colorectal tumorigenesis. High circulating levels of MMP-2 and MMP-9 in colorectal cancer patients may serve as potential indicators for angiogenesis and liver metastasis (Xu et al., 2007).

Strong expression of MMP-2 and MMP-9 has been related to poor survival of colorectal cancer patients. The expression of TIMP-2 has been associated with both a beneficial and a poor outcome and there is thus a need to further clarify the significance of TIMP-2, MMP-2 and MMP-9 in colorectal cancer (Hilska et al., 2007). Moreover, these expressions of *TIMP-2*, *MMP-2* and *MMP-9* have been implicated as risk factors for several carcinomas including cancers of colorectal, lung, oral cavity, gastric, prostate, pancreatic, breast (Bojanowska-Pozniak et al., 2007; Xu et al., 2007; Miao et al., 2003; Zhou et al., 2004).

Thus, this study analyzes the 5 SNPs of at the promoter and exon regions of the *TIMP-2*, *MMP-2* and *MMP-9* genes in colorectal cancer patients and their association with age, lymph node metastasis and differentiation and preoperative serum CEA and American Joint Committee on Cancer (AJCC) stage.

Materials and Methods

Subjects

A total of 347 colorectal cancer patients (195 males and 152 females), 33 inflammatory bowel disease (IBD) patients (25 males and 8 females) and 312 healthy controls in Korean were included in this analysis. Colorectal cancer patients were recruited from the Asan Medical Center, Seoul, Korea. Colorectal cancer was classified according to the tumor-node-metastasis classification of the American Joint Committee on Cancer (2002). Table 1 presents clinicopathological factors of colorectal cancer patients.

Genotyping

Genomic DNA was extracted from peripheral blood using a QIAamp Blood kit (Quiagen, Hilden, Germany). Genotyping of the *TIMP-2 -418G>C*, *TIMP-2 303G>A*, *MMP-2 -735C>T*, *MMP-2 -1575G>A* and *MMP-9 -1562C>T* were determined by the polymerase chain

reaction-restriction fragment length polymorphism (PCR-RFLP) method. For detection of the polymorphism, 10 μl reaction volume containing 15 ng of DNA was amplified with 10 mM Tris (pH 8.0), 40 mM KCl, 1.5 mM $MgCl_2$, 200 M dNTP, 5 pmol each primer and 0.38 unit Taq polymerase (Bioneer, Korea) on GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA).

The *TIMP-2 -418G>C* polymorphism was amplified using sense 5'-CGTCTCTTGTTGGCTGGTCA-3' and antisense 5'-CCTTCAGCTCGACTCTGGAG-3' primers to be digested with the restriction enzyme *BsoB*I (New England Biolabs, USA). The PCR conditions were initial denaturation of 2 min at 94C followed by 35 cycles of 30s at 94C, 30s at 59C, 30s at 72C, and a final extension at 72C for 7 min. The *TIMP-2 303G>A* polymorphism was amplified using sense 5'-TAGGAACAGCCCCACTTCTG-3' and antisense 5'-CCTCCTCGGCAGTGTGTG-3'primers to be digested with the restriction enzyme *Tsp*RI (New England Biolabs, USA). The PCR conditions were initial denaturation of 2 min at 94C followed by 35

cycles of 30s at 94C, 30s at 60C, 30s at 72C, and a final extension at 72C for 7 min. The *MMP-2 -735C>T* polymorphism was amplified using sense 5'-ATAGGGTAAACCTCCCCACATT-3' and antisense 5'-GGTAAAATGAGGCTGAGACCTG-3'primers to be digested with the restriction enzyme *Hin*fI (New England Biolabs, USA). The PCR conditions were initial denaturation of 5 min at 95C followed by 30 cycles of 30s at 95C, 30s at 60C, 30s at 72C, and a final extension at 72C for 8 min. The *MMP-2 -1575G>A* polymorphism was amplified using sense 5'-ACTGACTCTGGAAAGTCAGAGCA-3' and antisense 5'-GGCACAGGGTGAGGGGATGG-3'primers to be digested with the restriction enzyme *Tsp45*I (New England Biolabs, USA). The PCR conditions were initial denaturation of 3 min at 94C followed by 35 cycles of 30s at 94C, 30s at 64C, 30s at 72C, and a final extension at 72C for 5 min. The *MMP-9 -1562C>T* polymorphism was amplified using sense 5'-GCCTGGCACATAGTAGGCCC-3' and antisense 5'-CTTCCTAGCCAGCCGGCATC-3' primers to be digested with the restriction enzyme *Sph*I (New England Biolabs, USA). The PCR

conditions were initial denaturation of 4 min at 94C followed by 35 cycles of 20s at 94C, 20s at 63C, 30s at 72C, and a final extension at 72C for 7 min. Digested PCR products were electrophoresed in 8%(*TIMP-2* and *MMP-2*) and 5%(*MMP-9*) polyacrylamide gel and stained with ethidium bromide to visualize in UV.

Statistical analysis

The differences of allele frequencies and genotype distribution between colorectal cancer patients and controls were examined by the χ^2 test using SAS v.9.1 (SAS Institute, Cary, NC). Odds ratios (OR) with 95% CI were obtained. The *P*-values <0.05 were regarded as statistically significant. The Hardy-Weinberg equilibrium and linkage disequilibrium (LD) were analyzed using the R program v.2.9.0 (http://www.r-projects.org/). LD; the most frequently used is D' > 0.8 (Carlson et al., 2004). The PHASE program v.2.0.1 was used to infer haplotypes.

Results

The genotype and allele distributions of the *TIMP-2, MMP-2* and *MMP-9* polymorphisms in colorectal patients and healthy controls are shown in Table 2. Each genotype and allele frequency of the *TIMP-2, MMP-2* and *MMP-9* polymorphisms in colorectal patients and healthy controls were in the Hardy-Weinberg equilibrium. The homozygous *TIMP-2 -418*G/*G* and *303*G/*G (Ser101Ser)* and *MMP-9 -1562*C/*C* genotypes were significantly higher in colorectal cancer patients than in healthy controls (p = 0.024, OR = 1.4, 95% CI = 1.05 - 1.98; p <.0001, OR = 4.0, 95% CI = 2.67 - 6.02; p = 0.026, OR = 1.5, 95% CI = 1.05 - 2.21, respectively). *MMP-2 -1575*G/*G* genotype was significantly higher in colorectal cancer patients than in healthy controls (p = 0.007, OR = 0.6, 95% CI = 0.42 - 0.87). There was no significant difference in the genotype frequency of the *MMP-2 -735C>T* (Table 2).

Haplotype of *TIMP-2 -418G-303G* revealed increased susceptibility to colorectal cancer as compared with controls while, that of *MMP-2 -735C-1575G* revealed decreased susceptibility to colorectal cancer as compared with controls (p <.0001, OR = 2.0, 95% CI = 1.55 - 2.51; p = 0.002, OR = 0.7, 95% CI = 0.56 - 0.88; Table 4).

The colorectal cancer patients who carried the *TIMP-2 -418*G/*G* and *303*G/*G* were significantly increased compared with controls (59.7% vs. 41.7%; p <.0001). Conversely, the colorectal cancer patients who carried the *TIMP-2 -418*C/*G, *C/*C* and *303*A/*G, *A/*A* were significantly decreased compared with controls (3.4% vs. 16.3%; p <.0001; Figure 1). The colorectal cancer patients who carried the *TIMP-2 -418*G/*G, 303*G/*G* and *MMP-9 -1562*C/*C* were significantly increased compared with controls (48.8% vs. 29.1%; p <.0001). Conversely, the colorectal cancer patients who carried the *TIMP-2 -418*C/*G, *C/*C, TIMP-2 303*A/*G, A/*A* and *MMP-9 -1562*C/*C* were significantly decreased compared with controls (3.0% vs. 10.3%; p = 0.0001; Figure 2). The colorectal cancer patients who

carried the *MMP-2 -735 *C/*C* and *-1575*G/*G* were significantly decreased compared with controls (35.4% vs. 46.5%; p = 0.004; Figure 3). Association of *TIMP-2 -418G>C*, *TIMP-2 303G>A*, *MMP-2 -1575G>A* and *MMP-9 -1562C>T* genotypes with age, lymph node metastasis, differentiation, preoperative serum CEA and AJCC stage was analyzed in colorectal cancer patients. No significant association between the *TIMP-2 -418G>C*, *TIMP-2 303G>A*, *MMP-2 -1575G>A* and *MMP-9 -1562C>T* genotypes and the age, lymph node metastasis, differentiation, PRCEA and AJCC stage was observed in colorectal cancer (Table 5). The linkage disequilibrium (LD) between 2SNPs of *TIMP-2* as well as *MMP-2* was not observed (Figure 4).

The genotype and allele distributions of the *TIMP-2, MMP-2* and *MMP-9* polymorphisms in inflammatory bowel disease (IBD) patients and healthy controls are shown in Table 3. There were no significant difference in the genotypes and allelic frequencies of the *TIMP-2 -418G>C, TIMP-2 303G>A, MMP-2 -735C>T, MMP-2 -1575G>A* and *MMP-9 -1562C>T* (Table 3).

Discussion

TIMP-2 -418G>C polymorphism has been identified in the promoter region of the *TIMP-2* gene. The presence of the *G* allele has been shown to be associated with increased gene expression, possibly because it favors the binding of the Sp1 transcription factor on a consensus sequence in the promoter region of the *TIMP-2* gene (Vairaktaris et al., 2007). *MMP-2 -735C>T* is upstream from the transcriptional start site of the *MMP-2* gene and has a significant effect on the transcriptional activity. The transition of *C* to *T* located at nucleotide -735 of the promoter region of *MMP-2* destroys a Sp1-binding site (Kang et al., 2008). *MMP-2 -1575G>A* alters binding of oestrogen receptor (Clark et al., 2008). *MMP-9 -1562C>T* affects transcriptional activity (Peres et al., 2005). A 9-bp sequence (GCGCA*C/T*GCC, -1567 to -1559) containing the *MMP-9 -1562C>T* site has been suggested to function as an important regulatory element by serving as a binding site for a transcription repressor protein (Zhang et al., 1999).

The levels of mRNA of TIMP-2 and MMP-2 were significantly elevated in colorectal tissue samples in comparison to adjacent normal tissue samples in Czech (Pesta et al., 2007). MMP-2 activity and expression level were much higher in colorectal carcinoma tissues than in normal tissues in China (Li et al., 2005). The expression of MMP-2 and MMP-9 was not detected in colorectal normal tissues but it was significantly increased in colorectal cancer tissues in China (Wu et al., 2008). High expression of MMP-2 in the malignant epithelium as well as in the surrounding stroma was associated with reduced survival of colon cancer patients in Finland (Hilska et al., 2007).

In this study, the homozygous *TIMP-2 -418*G/*G* genotype was significantly increased in colorectal cancer patients compared to healthy controls (p = 0.024, OR = 1.4, 95% CI = 1.05 - 1.98). Although there was not statistically significant, the frequency of *TIMP-2 -418*G/*G* genotype was higher in gastric cancer than in healthy controls (81.7% vs 78.8%; Wu et al., 2007) and was higher in breast cancer patients than in controls (69.5% vs 63.3%; Zhou et al., 2004). In

this study, the homozygous *TIMP-2 303*G/*G* genotype was significantly increased in colorectal cancer patients compared to healthy controls (p <.0001, OR = 4.0, 95% CI = 2.67 - 6.02). Although there was not statistically significant, the frequency of *TIMP-2 303*G/*G* genotype was higher in gastric cancer patients than in controls (86.1% vs 78.7%; Kubben et al., 2006). Increased TIMP-2 expression has been reported in the colorectal cancer (Li et al., 2005). Increased TIMP-2 mRNA levels have been correlated with tumor stage, lymph node metastasis, and shortened survival in patients with carcinomas of colon, breast, and bladder (Nemeth et al., 1996). TIMP-2 expression appears to have a tumor-promoting role in prostate cancer (Ross et al., 2003). So, these results suggest that *TIMP-2 -418*G/*G* and *TIMP-2 303*G/*G* genotypes were positively correlated with colorectal cancer.

Although there was not statistically significant, the frequency of *MMP-2 -735*C/*C* genotype was lower in colorectal cancer than in healthy controls in this study (p = 0.106, OR = 0.8, 95% CI = 0.57 -

1.06). Although there was not statistically significant, the frequency of *MMP-2 -735*C/*C* genotype was higher in adenomyosis cases than in controls (54.5% vs 60.8%; Kang et al., 2008). In this study, *MMP-2 -1575*G/*G* genotypes was associated with reduced risk between colorectal cancer patients and healthy controls (p = 0.007, OR = 0.6, 95% CI = 0.42 - 0.87). It has been reported that the frequency of *MMP-2 -1575*G/*G* genotype was significantly higher in colorectal cancer than in healthy controls in Chinese (p = 0.03, OR = 1.96, 95% CI = 1.06 - 3.64; Xu et al. 2007). Moreover, MMP-2 expression level was not only associated with the development of colorectal cancer, but also played a very important role in the process of colorectal cancer invasion and metastasis (Li et al., 2005). So, these results suggest that *MMP-2 -1575*G/*G* genotypes was negatively correlated with colorectal cancer.

In this study, *MMP-9 -1562C>T* was shown to have significant association with colorectal cancer. *MMP-9 -1562*C/*C* genotypes was associated with increased risk between colorectal cancer patients and

healthy controls (p = 0.026, OR = 1.5, 95% CI = 1.05 - 2.21). It was reported that *MMP-9 -1562C>T* polymorphism was not involved in the process of colorectal carcinogenesis, as indicated by the consistent frequency of allele and genotype in colorectal cancer patients and controls (p = 0.48, OR = 1.24, 95% CI = 0.69 - 2.22; Xu et al., 2007). Moreover, over expression of MMP-9 has been demonstrated in colorectal cancer and high expression of MMP-9 has been reported to be induced in the esophageal cancer tissue (Mroczko et al., 2008; Zucker et al., 2004). So, these results suggest that *MMP-9 -1562*C/*C* genotype was positively correlated with colorectal cancer.

In the Table 6, there are the genotype and allele frequencies of *TIMP-2, MMP-2* and *MMP-9* genes SNPs in other ethnic populations. The allele frequency of *TIMP-2 -418*G* (0.766) in Korean differs from that in Chinese (0.824), Taiwan (0.871), Caucasian (0.997) and German (0.964) (Chen et al., 2007; Kubben et al., 2006; Vairaktaris et al., 2007 Wu et al., 2007). The allele frequency of *TIMP-2 303*G* (0.811) in Korean differs from Caucasian (0.890) and German (0.890) (Krex et al.,

2003; Kubben et al., 2006). The allele frequency of *MMP-2 -735*C* (0.766) in Korean was found to similar to that in Chinese (0.779), but differs from that in Czech (0.847) (Kang et al., 2008; Vasku et al., 2009). The allele frequency of *MMP-2-1575*G* (0.891) in Korean was found to be more frequent than in Czech (0.735) (Vasku et al., 2009).The allele frequency of *MMP-9 -1562*C* (0.862) in Korean was similar to that Japanese (0.840), Chinese (0.891), Caucasian (0.846) (Chen et al., 2007; Kubben et al., 2006; Minematsu et al., 2001).

In conclusion, this study suggests that the *TIMP-2 -418*G/*G* and *303*G/*G* and *MMP-9 -1562*C/*C* genotypes reveal increased susceptibility to colorectal cancer. *MMP-2 -1575*G/*G* genotypes reveal decreased susceptibility to colorectal cancer. Moreover, the haplotype of *TIMP-2 -418G-303G* enhances susceptibility to colorectal cancer and the haplotype of *MMP-2 -735C-1575G* reduces the susceptibility of colorectal cancer.

Table 1. Clinicopathological classification of colorectal cancer

Characteristics	Colorectal Cancer n = 347 (%)
Gender	
Male	195 (56.2)
Female	152 (43.8)
Age (year)	
<40	35 (10.1)
40-55	128 (36.9)
>55	184 (53.0)
Mean (range)	58.6 (23-82)
Lymph node metastasis	
positive	107 (32.3)
negative	224 (67.7)
Differentiation	
WD + MD	286 (88.3)
PD	38 (11.7)
PRCEA (ng/ml)	
≤ 6	260 (76.7)
> 6	79 (23.3)
AJCC stage	
Stage Ⅰ + Ⅱ	218 (63.9)
Ⅲ + Ⅳ	123 (36.1)

WD: well differentiated, MD: moderately differentiated, PD: poorly differentiated
PRCEA: Preoperative serum CEA
AJCC: American Joint Committee on Cancer

Table 2. Genotype and allele frequencies of *TIMP-2, MMP-2* and *MMP-9* genes in colorectal cancer

SNPs	Colorectal Cancer n= 347 (%)	Controls n= 312 (%)	*P*	OR(95% CI)
TIMP-2				
c.-418G>C				
*G/G	234 (67.4)	184 (59.0)	0.024	1.4 (1.05-1.98)
*C/G	98 (28.2)	110 (35.2)		
*C/C	15 (4.3)	18 (5.8)		
*G allele	0.816	0.766		
c.303G>A (exon3; *Ser101Ser*)				
*G/G	308 (88.8)	207 (66.3)	<.0001	4.0 (2.67-6.02)
*A/G	38 (10.9)	92 (29.5)		
*A/A	1 (0.3)	13 (4.2)	0.0006	0.1 (0.01-0.51)
*G allele	0.942	0.811		
MMP-2				
c.-735C>T				
*C/C	184 (53.0)	185 (59.3)		
*C/T	136 (39.2)	108 (34.6)		
*T/T	27 (7.8)	19 (6.1)		
*C allele	0.726	0.766		
c.-1575G>A				
*G/G	245 (70.6)	249 (79.8)	0.007	0.6 (0.42-0.87)
*A/G	92 (26.5)	58 (18.6)		
*A/A	10 (2.9)	5 (1.6)		
*G allele	0.839	0.891		
MMP-9				
c.-1562C>T				
*C/C	283 (81.5)	232 (74.4)	0.026	1.5 (1.05-2.21)
*C/T	62 (17.9)	74 (23.7)		
*T/T	2 (0.6)	6 (1.9)		
*C allele	0.905	0.862		

P: colorectal cancer patients vs. controls

Table 3. Genotype and allele frequencies of *TIMP-2*, *MMP-2* and *MMP-9* genes in IBD

SNPs	IBD n= 33 (%)	Controls n= 312 (%)	*P*	OR(95% CI)
TIMP-2				
c.-418G>C				
*G/G	23 (69.7)	184 (59.0)		
*C/G	9 (27.3)	110 (35.2)		
*C/C	1 (3.0)	18 (5.8)		
*G allele	0.833	0.766		
c.303G>A (exon3; *Ser101Ser*)				
*G/G	26 (78.8)	207 (66.3)		
*A/G	7 (21.2)	92 (29.5)		
*A/A	0 (0.0)	13 (4.2)		
*G allele	0.894	0.811		
MMP-2				
c.-735C>T				
*C/C	19 (57.6)	185 (59.3)		
*C/T	10 (30.3)	108 (34.6)		
*T/T	4 (12.1)	19 (6.1)		
*C allele	0.727	0.766		
c.-1575G>A				
*G/G	27 (81.8)	249 (79.8)		
*A/G	5 (15.2)	58 (18.6)		
*A/A	1 (3.0)	5 (1.6)		
*G allele	0.894	0.891		
MMP-9				
c.-1562C>T				
*C/C	29 (87.9)	232 (74.4)		
*C/T	3 (9.1)	74 (23.7)		
*T/T	1 (3.0)	6 (1.9)		
*C allele	0.924	0.862		

P: colorectal cancer patients vs. controls

Table 4. Haplotype frequencies of *TIMP-2* and *MMP-2* in colorectal cancer patients and controls

Haplotype	Colorectal Cancer	Controls	*P*	OR (95%CI)
TIMP-2 -418G>C-303G>A				
G - G	0.769	0.630	<.0001	2.0 (1.55-2.51)
C - G	0.173	0.181		
G - A	0.047	0.136	<.0001	0.3 (0.20-0.47)
C - A	0.011	0.053	<.0001	0.2 (0.08-0.42)
MMP-2-735C>T-1575G>A				
C - G	0.593	0.673	0.002	0.7 (0.56-0.88)
T - G	0.246	0.218		
C - A	0.133	0.093	0.02	1.5 (1.07-2.14)
T - A	0.028	0.016		

Table 5. Comparison between the genotype of *TIMP-2 -418G>C*, *303G>A*, *MMP-9 -1562C>T* and the genotype of *MMP-2 -1575G>A* in colorectal cancer patients, stratified by clinicopathological factors

	Genotype frequency		colorectal cancer
	TIMP-2 -418**G/***G* and *TIMP-2 303***G/***G* and *MMP-9 -1562***C/***C*	*MMP-2 -1575***G/***G*	
Gender			
Male	89 (53.6)	137 (55.9)	195 (56.2)
Female	77 (46.4)	108 (44.1)	152 (43.8)
Age (year)			
<40	17 (10.2)	24 (9.8)	35 (10.1)
40-55	61 (36.7)	94 (38.4)	128 (36.9)
>55	88 (53.0)	127 (51.8)	184 (53.0)
Lymph node metastasis			
positive	55 (34.8)	81 (34.9)	107 (32.3)
negative	103 (65.2)	151 (65.1)	224 (67.7)
Differentiation			
WD + MD	137 (87.8)	45 (84.9)	286 (88.3)
PD	19 (12.2)	8 (15.1)	38 (11.7)
PRCEA (ng/ml)			
≤ 6	62 (70.5)	184 (77.0)	260 (76.7)
> 6	26 (29.5)	55 (23.0)	79 (23.3)
AJCC stage			
Stage Ⅰ + Ⅱ	107 (65.6)	74 (66.7)	218 (63.9)
Ⅲ + Ⅳ	56 (34.4)	37 (33.3)	123 (36.1)

WD: well differentiated, MD: moderately differentiated, PD: poorly differentiated
PRCEA: Preoperative serum CEA, AJCC: American Joint Committee on Cancer

Table 6. Genotype and allele frequencies of *TIMP-2*, *MMP-2* and *MMP-9* genes in other populations

Populations	n	Genotype frequency			Allele	Reference
TIMP-2 -418G>C		*G/*G	*C/*G	*C/*C	*G	
Korean	312	184(59.0)	110(35.2)	18(5.8)	0.766	This study
Chinese	128	87(68.0)	37(28.9)	4(3.1)	0.824	Chen et al., 2007
Taiwan	283	223(78.8)	47(16.6)	13(4.6)	0.871	Wu et al., 2007
Caucasian	169	168(99.4)	1(0.6)	0(0.0)	0.997	Kubben et al., 2006
German	56	52(92.9)	4(7.1)	0(0.0)	0.964	Vairaktaris et al., 2007
TIMP-2 303G>A		*G/*G	*A/*G	*A/*A	*G	
Korean	312	207(66.3)	92(29.5)	13(4.2)	0.811	This study
Caucasian	169	133(78.7)	35(20.7)	1(0.6)	0.890	Kubben et al., 2006
German	41	32(78.0)	9(22.0)	0(0.0)	0.890	Krex et al., 2003
MMP-2 -735C>T		*C/*C	*C/*T	*T/*T	*C	
Korean	312	185(59.3)	108(34.6)	19(6.1)	0.766	This study
Chinese	324	197(60.8)	111(34.3)	16(4.9)	0.779	Kang et al., 2008
Czech	196	142(72.4)	48(24.5)	6(3.1)	0.847	Vasku et al., 2009
MMP-2 -1575G>A		*G/*G	*A/*G	*A/*A	*G	
Korean	312	249(79.8)	58(18.6)	5(1.6)	0.891	This study
Czech	196	103(52.6)	82(41.8)	11(5.6)	0.735	Vasku et al., 2009
MMP-9 -1562C>T		*C/*C	*C/*T	*T/*T	*C	
Korean	312	232(74.4)	74(23.7)	6(1.9)	0.862	This study
Japanese	94	68(72.3)	22(23.4)	4(4.3)	0.840	Minematsu et al., 2001
Chinese	128	101(78.9)	26(20.3)	1(0.8)	0.891	Chen et al., 2007
Caucasian	169	120(71.0)	46(27.2)	3(1.8)	0.846	Kubben et al.,2006

Colorectal cancer n= 347 (%)	*TIMP-2 -418*G/*G*	*TIMP-2 -418*C/*G, -418*C/*C*
*TIMP-2 303*G/*G*	[1]**207 (59.7)**	**101 (29.1)**
*TIMP-2 303*A/*G, 303*A/*A*	[2]**27 (7.8)**	[3]**12 (3.4)**

Controls n= 312 (%)	*TIMP-2 -418*G/*G*	*TIMP-2 -418*C/*G, -418*C/*C*
*TIMP-2 303*G/*G*	[1]**130 (41.7)**	**77 (24.7)**
*TIMP-2 303*A/*G, 303*A/*A*	[2]**54 (17.3)**	[3]**51 (16.3)**

P: colorectal cancer vs. controls
1. OR = 2.1 (95% CI = 1.52-2.82)
2. OR = 0.4 (95% CI = 0.25-0.66)
3. OR = 0.2 (95% CI = 0.10-0.35)

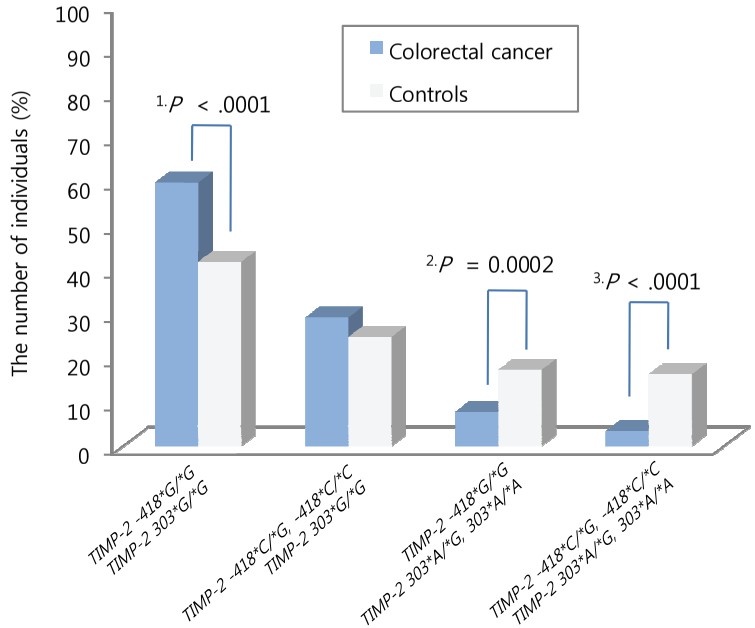

Figure 1. *TIMP-2 -418G>C* and *303G>A* combined genotype.

Colorectal cancer n= 347 (%)	TIMP-2			
	-418*G/*G 303*G/*G	-418*G/*G 303*A/*G, 303*A/*A	-418*C/*G ,-418*C/*C 303*G/*G	-418*C/*G, -418*C/*C, 303*A/*G, 303*A/*A
MMP-9 -1562*C/*C	[1]169 (48.8)	[2]22 (6.2)	82 (23.5)	[3]10 (3.0)
MMP-9 -1562*C/*T, -1562*T/*T	39 (11.1)	[4]5 (1.4)	18 (5.3)	[5]2 (0.7)

Controls n= 312 (%)	TIMP-2			
	-418*G/*G 303*G/*G	-418*G/*G 303*A/*G, 303*A/*A	-418*C/*G ,-418*C/*C 303*G/*G	-418*C/*G, -418*C/*C, 303*A/*G, 303*A/*A
MMP-9 -1562*C/*C	[1]91 (29.1)	[2]46 (14.8)	63 (20.2)	[3]32 (10.3)
MMP-9 -1562*C/*T, -1562*T/*T	31 (10.0)	[4]16 (5.1)	22 (7.0)	[5]11 (3.5)

P: colorectal cancer vs. controls
1. OR = 2.3 (95% CI = 1.67-3.18)
2. OR = 0.4 (95% CI = 0.23-0.67)
3. OR = 0.3 (95% CI = 0.13-0.54)
4. OR = 0.3 (95% CI = 0.10-0.75)
5. OR = 0.2 (95% CI = 0.03-0.72)

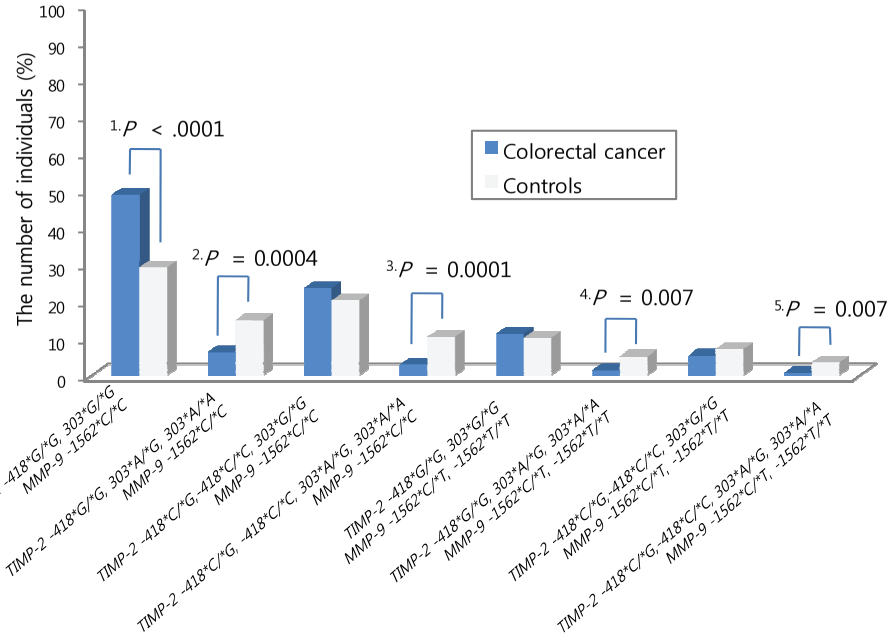

Figure 2. *TIMP-2 -418G>C, 303G>A* and *MMP-9 -1562C>T* combined genotype.

Colorectal cancer n= 347 (%)	*MMP-2 -735*C/*C*	*MMP-2 -735*C/*T, -735*T/*T*
*MMP-2 -1575*G/*G*	[1.]**123 (35.4)**	**122 (35.2)**
*MMP-2 -1575*A/*G, -1575*A/*A*	**61 (17.6)**	**41 (11.8)**

Controls n= 312 (%)	*MMP-2 -735*C/*C*	*MMP-2 -735*C/*T, -735*T/*T*
*MMP-2 -1575*G/*G*	[1.]**145 (46.5)**	**104 (33.3)**
*MMP-2 -1575*A/*G, -1575*A/*A*	**40 (12.8)**	**23 (7.4)**

P: colorectal cancer vs. controls
[1.] OR = 0.6 (95% CI = 0.46-0.86)

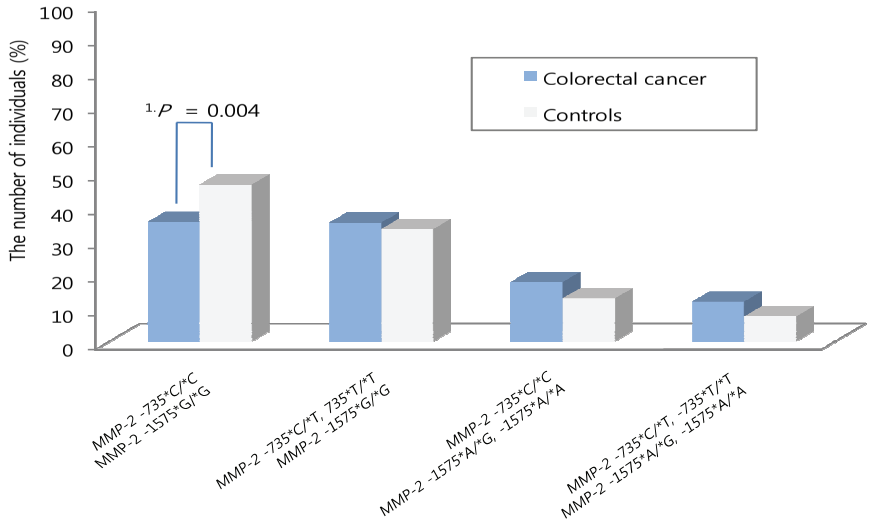

Figure 3. *MMP-2 -735C>T* and *-1575G>A* combined genotype.

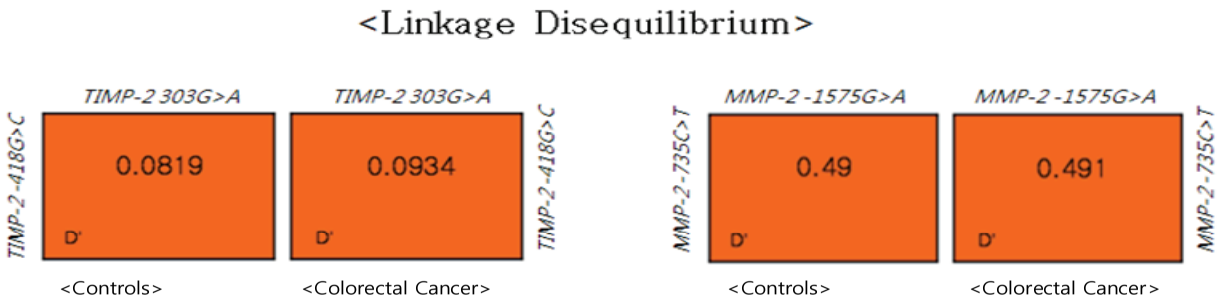

Figure 4. Linkage disequilibrium coefficient | D' | between 2SNPs of *TIMP-2* and between 2SNPs of *MMP-2* in the Korean population.

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Abstract

Association between Tissue Inhibitor of Metalloproteinase (TIMP)-2, Matrix Metalloproteinase (MMP)-2 and MMP-9 Polymorphisms and Colorectal Cancer

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Tissue inhibitor of metalloproteinase-2 (TIMP-2), matrix metalloproteinase-2 (MMP-2) and MMP-9 participate in the degeneration of the extracellular matrix and associated with malignant tumor growth, invasion and metastasis. And these expressions of *TIMP-2*, *MMP-2* and *MMP-9* have been implicated as risk factors for several carcinomas including cancers of colorectal, lung, oral cavity, gastric, prostate, pancreatic and breast. Thus, this study analyzes the 5 SNPs of at the promoter and exon regions of the *TIMP-2*, *MMP-2* and *MMP-9* genes in colorectal cancer patients.

A total of 347 patients with colorectal cancer, 33 patients with inflammatory bowel disease (IBD) and 312 healthy controls were included in this case-control study. The genotyping of *TIMP-2 -418G>C*, *TIMP-2 303G>A*, *MMP-2 -735C>T*, *MMP-2 -1575G>A* and

MMP-9 -1562C>T was done using the PCR-RFLP method. The homozygous *TIMP-2 -418*G/*G* and *303*G/*G (Ser101Ser)* genotypes, and haplotype of *TIMP-2 -418G-303G* were significantly higher in colorectal cancer patients than in healthy controls (p = 0.024, OR = 1.4, 95% CI = 1.05 - 1.98; p <.0001, OR = 4.0, 95% CI = 2.67 - 6.02; p <.0001, OR = 2.0, 95% CI = 1.55 - 2.51, respectively). And the homozygous *MMP-9 -1562*C/*C* genotype was significantly higher in colorectal cancer patients than in healthy controls (p = 0.026, OR = 1.5, 95% CI = 1.05 - 2.21). While, the homozygous *MMP-2 -1575*G/*G* genotype and haplotype of *MMP-2 -735C-1575G* were significantly lower in colorectal cancer patients than in healthy controls (p = 0.007, OR = 0.6, 95% CI = 0.42 - 0.87; p = 0.002, OR = 0.7, 95% CI = 0.56 - 0.88, respectively). No significant association between the *TIMP-2 -418G>C*, *TIMP-2 303G>A*, *MMP-2 -1575G>A* and *MMP-9 -1562C>T* genotypes and the age, lymph node metastasis, differentiation, PRCEA and AJCC stage was observed in colorectal cancer. There were no significant difference in the genotypes and allelic frequencies of the *TIMP-2 -418G>C, TIMP-2 303G>A, MMP-2 -735C>T, MMP-2 -1575G>A* and *MMP-9 -1562C>T* in IBD patients compared to healthy controls.

In conclusion, the homozygous *TIMP-2 -418*G/*G*, *TIMP-2 303*G/*G, MMP-2 -1575*G/*G* and *MMP-9 -1562*C/*C* genotypes were associated with colorectal cancer, but were not associated with the age, lymph node metastasis, differentiation, PRCEA and AJCC stage in colorectal cancer.

감사의 글

본 논문을 완성하기까지 끊임없이 힘을 주신 주위의 많은 분들께 감사의 마음을 전합니다.

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