RAPID COMMUNICATION

Role of matrix metalloproteinase, tissue inhibitor of metalloproteinase and tumor necrosis factor-α single nucleotide gene polymorphisms in inflammatory bowel disease

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Abstract

AIM: To study the (functional) relevance of single nucleotide polymorphisms (SNPs) in genes encoding matrix metalloproteinases (MMP)-1, -2, -3, -9, tissue inhibitors of metalloproteinases (TIMP)-1, -2 and tumor necrosis factor (TNF)-α in the etiopathogenesis of inflammatory bowel diseases (IBD), that may enhance susceptibility and/or disease severity.

METHODS: Genomic DNA from 134 Crohn's disease (CD), 111 ulcerative colitis (UC) patients and 248 control subjects was isolated from resected intestinal tissue or blood. Allelic composition at SNP loci was determined by PCR-RFLP or tetra primer ARMS PCR.

RESULTS: The TIMP-1 genotype TT in women and T in men at SNP +372 T/C was found to increase CD susceptibility (39% *vs* 23.8%, *P* = 0.018 and 67.9% *vs* 51.6%, *P* = 0.055, respectively), while women with this genotype were less prone to development of fistulae during follow-up (41.4% *vs* 68.3%, *P* = 0.025). Male IBD or CD patients carrying the TIMP-1 +372 T-allele expressed lower levels of TIMP-1 in surgically resected macroscopically inflamed tissue (0.065 < *P* < 0.01). The 5T5T genotype at MMP-3 SNP -1613 5T/6T increased the chance of stenotic complications in CD during follow-up (91.2% *vs* 71.8%, *P* = 0.022) but seemed to protect against colonic involvement of this disease at first endoscopic/radiologic examination (35.3% *vs* 59.5%, *P* = 0.017).

CONCLUSION: Allelic composition at the examined

SNPs in genes coding for TIMP-1 and MMP-3 affect CD susceptibility and/or phenotype, i.e., fistulizing disease, stricture pathogenesis and first disease localisation. These findings reinforce the important role of these proteins in IBD.

INTRODUCTION

Crohn's disease (CD) is characterised by chronic, patchy, transmural inflammation of the gastrointestinal tract, predominantly in the ileocecal area, while ulcerative colitis (UC) is manifested by chronic, continuous, rather superficial inflammation of the mucosal layers of the colon[1,2]. The incidence and prevalence of both CD and UC have increased in the Western population since the second World War[3,4], and lately also increased in developing industrialising countries. Although there has been much controversy regarding etiology and pathogenesis of both forms of inflammatory bowel disease (IBD), recent evidence points to an exaggerated immune response to enteric bacterial flora in genetically susceptible individuals. Based on a higher disease concordance in monozygotic *vs* dizygotic twins[5], a higher frequency of IBD in certain families and ethnic groups[6,7], the association of IBD with genetic disorders like Turner's and Hermansky-Pudlak syndrome[8,9], the presence of a genetic component in IBD is evident. Indeed, large-scale genome-wide linkage studies have mapped several regions of the human genome to IBD, i.e., 16q12 (IBD1),

12q13 (IBD2), 6p21 (IBD3), 14q11 (IBD4), 19p13 (IBD5), 5q31-q33 (IBD6) and Xq21.3[10-15] and subsequent research has identified several CD predisposing mutations in the IBD1 gene encoding NOD2[16]. However, the different chromosomal locations found to be associated with IBD in these studies suggest disease heterogeneity: different sets of disease predisposing mutations may lead to a similar clinical outcome. This is corroborated by evidence obtained from animal models, where distinct genetic manipulations, for instance deletion of the DNA encoding TCRα, IL-10 or TNF-α 3'UTR AU repeat motifs, all lead to ileitis and/or colitis[17-19]. Therefore, genes on other loci, not identified in the studies mentioned above, may also contribute to IBD susceptibility and worthy considering in this respect are the matrix metalloproteinases (MMPs) and their natural inhibitors, the tissue inhibitors of metalloproteinases (TIMPs). The MMPs constitute a group of neutral, Ca- and Zn-activated endoproteinases and are involved in physiological matrix turnover during embryogenesis, angiogenesis, *etc*[20]. Production is tightly regulated at the transcriptional and post-transcriptional levels, and excessive MMP-mediated tissue destruction is prevented by strictly regulated activation mechanisms of the latent pro-enzyme and inhibition of the active enzyme in a 1:1 stoichiometry by TIMPs. Recently, several functional single nucleotide polymorphisms (SNPs) in the genes encoding MMPs and TIMPs have been described. The insertion of an additional guanosine residue at -1607 in the promoter of MMP-1 creates a PEA3 consensus sequence next to an AP-1 binding site up-regulating promoter activity, while the insertion of an additional thymidine at -1613 of the MMP-3 promoter results in decreased mRNA transcription[21,22]. The -1306 C/T transition in the promoter of MMP-2 results in decreased binding affinity for stimulating protein Sp1, leading to decreased mRNA transcription[23]. In contrast, the -1562 C/T transition in the promoter of MMP-9 results in the removal of a binding site for an unknown repressor protein, thus elevating transcription[24]. In TIMP-1 and -2 SNPs have been found in the exon part of the genes (+372 T/C and +303 G/A, respectively). Although no effect on transcriptional activity/mRNA stability was observed, these SNPs might serve as markers in association studies[25]. Both MMP and TIMP expression are affected by TNF-α and this pro-inflammatory cytokine is known to play a pivotal role in IBD, particularly CD but also UC, as demonstrated by impressive clinical improvement following anti-TNF-α antibody infliximab administration[26,27]. The G/A transition at -308 in the TNF-α promoter might result in increased levels of circulating TNF-α protein[28], thus inducing extra MMPs and/or TIMPs. Of note, the gene encoding TNF-α is mapped to the 6p21 IBD3 region, while the MMP and TIMP genes have not been mapped to any known IBD region (MMP-1, -3: 11q22-q23; MMP-2: 16q13; MMP-9: 20q11.2-q13.1; TIMP-1: Xp11.3-p11.23 and TIMP-2: 17q25). Conceivably, direct and indirect SNP-linked overproduction of MMPs and/or down-regulation of TIMPs, would result in net destruction of tissue, impairment of intestinal barrier function, influx of bacteria and consequently excessive immune response, thus predisposing to or worsening IBD. Therefore, we analysed the genotype distributions at these SNP loci of the genes encoding MMP-1, -2, -3, -9, TIMP-1, -2 and TNF-α in CD, UC and controls. Recently, we measured MMP and TIMP protein/activity levels in a large group of resected intestinal IBD tissues (Meijer *et al*[32], submitted) and here the expression data in a subgroup of which we also had DNA, are correlated to MMP, TIMP and TNF genotypes.

MATERIALS AND METHODS

Study design

Surgically resected intestinal mucosa from predominantly Dutch Caucasian patients with CD (n = 134, 40% male, median age at surgery 36.3 years, range 11.6-78.7 years) or UC [n = 111, 42% male, 37.8 (15.9-81.9) years], was collected in the period 1983-2002 at the department of Pathology, LUMC and stored at -70℃. The control group consisted of 79 patients with colorectal carcinoma [CRC, macroscopically normal tissue obtained at least 10 cm away from evident neoplasia, 43% male, median age at surgery 56.4 (19.0-85.0) years] and 169 healthy volunteers [37% male, age at blood collection date 33.3 (18.2-72.9) years], recruited among spouses of patients from the out-patient clinic and through advertisement. Informed consent from participants and approval of the LUMC ethics committee for the study protocol was obtained[29]. In both the IBD and control groups, more than 95% of the participants were of Caucasian origin. Resected tissue was homogenised with a Turrax device, blood was centrifuged and genomic DNA was isolated using the salting out method[30] and reconstituted to 10 ng/μL in 0.01 mol/L Tris/0.1 mmol/L EDTA, pH = 7.5. Differential diagnosis of CD or UC was established by routine clinical, radiological and histological findings. Age at onset, localisation at first endoscopy/radiology and development of fistulae and stenotic processes in a subset of CD patients (n = 123) were recorded in medical files. The measurement of myeloperoxidase was according to the procedure described by Kruidenier *et al*[31], while the MMP and TIMP protein/activity levels in IBD and CRC control tissue were measured previously by our group (Meijer *et al*[32] and submitted). In brief, homogenates obtained from surgically resected tissue were appropriately diluted. The MMPs and TIMPs antigen levels were measured by ELISAs (MMP-2, -9, TIMP-1, -2) or by highly sensitive bio-immuno activity assays (BIA) involving the conversion of chromogenic peptide S-2444 by MMP-activated pro-urokinase (MMP-1, -3), with all BIAs performed in the presence of APMA to account for total MMP antigen levels. Allelic composition at the SNPs of interest was determined by PCR-RFLP (MMP-1, -3, -9, TIMP-1, -2) or tetra primer ARMS PCR (MMP-2), as described previously[32,33]. Differences between groups were assessed by Chi-square, Kruskal-Wallis or Mann-Whitney *U* tests, as indicated. Statistical significance was reached if two-tailed *P* value ≤ 0.05.

RESULTS

Allelic composition at SNP loci of MMP, TIMP and TNF genes

Between IBD and controls, no significant differences in genotype distribution were found at -1607 1G/2G and

Table 1 Genotype distributions at SNP loci in IBD patients compared to controls

Protein	SNP	Genotype	IBD (% of patients)	Controls (% of individuals)	Allele	IBD (% of total)	Controls (% of total)
MMP-1	-1607 1G/2G	1G1G	31	26.6	1G	55.4	51.4
	11q22-q23	1G2G	49	49.6	2G	44.6	48.6
		2G2G	20.1	23.8			
MMP-2	-1306 C/T	CC	57.7	61.9	C	75.5	76.8
	16q13	CT	35.6	29.9	T	24.5	23.2
		TT	6.7	8.2			
MMP-3	-1613 5T/6T	5T5T	29.2	27.9	5T	52.3	52
	11q23	5T6T	46.3	48.4	6T	47.7	48
		6T6T	24.6	23.8			
MMP-9	-1562 C/T	CC	72.4	69.7	C	85.6	84
	20q11.2-q13.1	CT + TT	27.6	30.3	T	14.4	16
♂, TIMP-1[1]	+372 T/C	T	61.6	51.6	T	61.6	51.6
	Xp11.3-p11.23	C	38.4	48.4	C	38.4	48.4
♀, TIMP-1[1]	+372 T/C	TT	31.2	23.8	T	52.8	50.7
	Xp11.3-p11.23	TC	43.3	53.6	C	47.2	49.3
		CC	25.5	22.5			
TIMP-2	+303 G/A	GG	77.5	78.7	G	88.3	89.1
	17q25	GA + AA	22.5	21.3	A	11.7	10.9
TNF-α	-308 G/A	GG	68.8	69.7	G	82.0	83.0
	6p21	GA + AA	31.4	30.3	A	18.0	17.0

n = 239-240/245 IBD patients *vs* n = 244/248 controls with successful genotype determinations. [1]For TIMP-1, ♂ these numbers are 99/101 *vs* 93/96 and for TIMP-1, ♀ 141/144 *vs* 151/152. Note: for MMP-9, TNF-α and TIMP-2 frequency of the homozygote mutant genotype (TT, AA and AA, respectively), was below 5% and these groups were combined with the corresponding heterozygote group, solely for the purpose of accurate statistical analysis. No statistically significant differences (Chi-Square testing) in genotype distributions or allele frequencies were found between IBD and controls.

Table 2 TIMP-1 genotype distribution at SNP + 372 C/T in CD patients compared to controls

Protein	Genotype	CD (% of patients)	Controls (% of individuals)	P^1	Allele	CD (% of total)	Controls (% of total)	P-value
TIMP-1, ♂	T	67.9	51.6	0.055	T	67.9	51.6	0.055
	C	32.1	48.4		C	32.1	48.4	
TIMP-1, ♀	TT	39	23.8	0.018	T	56.5	50.7	0.238
	TC	35.1	53.6		C	43.5	49.3	
	CC	26	22.5					

♂: n = 53/54 *vs* 93/96; ♀: n = 77/80 *vs* 151/152 successful genotype determinations, respectively. [1]P-value, Chi-Square test.

-1306 C/T of MMP-1 and -2 promoters, respectively (Table 1). Also, 1G MMP-1 (55.4 *vs* 51.4%) and C wild-type (75.5 *vs* 76.8%) MMP-2 allelic frequencies were similar in both groups. The MMP-3 and MMP-9 genotype distribution at -1613 5T/6T and -1562 C/T, respectively, were also similar. The TIMP-1 gene is located on the X-chromosome, thus the results are presented according to gender. In both men and women the T (T) genotype seems relatively abundant in IBD (men T 61.6 *vs* 51.6%; women TT 31.2 *vs* 23.8%) and especially in CD (men T 67.9 *vs* 51.6%, P = 0.055; women TT 39.0 *vs* 23.8%, P = 0.018, Table 2). No differences in genotype distribution were observed for TIMP-2 and TNF-α at +303 G/A and -308 G/A, respectively (Table 1). For all SNPs, genotype frequencies in the control group are similar to what was expected from the Hardy-Weinberg equilibrium, except for MMP-2 (CC, CT, TT: 61.9, 29.9, 8.2 observed *vs* 59.0, 35.7, 5.3% expected, χ^2 = 6.36, P < 0.05). Genotype and allelic frequencies for all SNPs examined were similar in CD *vs* UC and also in the healthy volunteers *vs* the carcinoma controls. As MMPs and TIMPs are involved in cancer and metastasis, all analyses were repeated with a control group consisting only of the healthy volunteers (n = 169), yielding similar results as mentioned above.

Effect of MMP and TNF-α SNPs on CD phenotype

The median age at onset of disease in 123 CD patients with a full medical record was 21.5 (range 0.3-61.5) years. Patients stratified according to genotype at the SNPs examined had similar ages at onset (Table 3). At first endoscopic/radiologic examination, in 53.3% of the patients colonic w/wo ileal involvement was evident. The MMP-3 genotype was associated with disease localisation (P = 0.04 for all three groups) and further analysis revealed a lower chance of colonic involvement at first endoscopy/radiology in patients with the 5T5T MMP-3 genotype (P = 0.017, 5T5T *vs* 5T6T and 6T6T combined). However, this genotype also conferred a major risk to development of stenotic complications: 91.2% of patients carrying the 5T5T genotype suffered from stenotic complications compared to 71.8% for the other genotypes (P = 0.022). The allelic polymorphisms at other SNP loci were not associated with disease localisation or stricture involvement. Of all CD patients, 80/123 or 65.0%

Table 3 Effect of MMP, TIMP and TNF-α allelic SNP composition on age at onset of disease, colon involvement at first endoscopic/radiologic examination and development of fistulae or stenotic strictures during follow-up [median 24.7 (range 3.3-58.5) yr] in CD

Protein	SNP	Genotype	Number of patients	Age (yr) at onset of disease, median (range)	Colonic involvement at first examination (% of patients)	Fistulae during follow-up (% of patients)	Strictures during follow-up (% of patients)
MMP-1	-1607 1G/2G	1G1G	38	20.7 (0.3-45.6)	52.6	71.1	84.2
		1G2G	54	22.4 (7.0-61.5)	50	63	75.9
		2G2G	26-27	21.0 (3.4-40.2)	57.7	63	70.4
MMP-2	-1306 C/T	CC	66-67	20.1 (5.8-61.5)	57.6	64.2	74.6
		CT+TT	52[1]	22.4 (0.3-50.1)	46.2	67.3	80.8
MMP-3	-1613 5T/6T	5T5T	34	22.4 (0.3-53.6)	35.3[a]	70.6	91.2[a]
		5T6T	54	19.3 (3.4-61.5)	63	66.7	74.1
		6T6T	30-31	23.5 (7.2-49.1)	53.3	58.1	67.7
MMP-9	-1562 C/T	CC	88-89	21.0 (0.3-61.5)	53.4	68.5	75.3
		CT+TT	30[1]	22.7 (7.2-45.5)	50	56.7	83.3
TIMP-1, ♂	+372 T/C	T	32	18.9 (0.3-48.3)	53.1	78.1	87.5
		C	17	18.5 (5.9-61.5)	52.9	76.5	94.1
TIMP-1, ♀	+372 T/C	TT	29	24.3 (7.0-56.8)	55.2	41.4[c]	69.0
		TC	22-23	23.5 (3.4-39.6)	45.5	69.6	78.3
		CC	18	22.2 (10.4-49.1)	55.6	66.7	55.6
TIMP-2	+303 G/A	GG	91-92	21.5 (0.3-61.5)	56.0	68.5	77.2
		GA+AA	27[1]	22.4 (3.4-56.8)	40.7	55.6	77.8
TNF-α	-308 G/A	GG	79-80	22.6 (0.3-61.5)	50.6	68.8	78.8
		GA+AA	39[1]	18.9 (3.4-45.5)	56.4	59.0	74.4

[1]Only 7, 1, 2 and 4 patients were carrying the MMP-2, -9, TIMP-2 or TNF-α mutant genotype, respectively, and were combined with the heterozygote group for statistical purposes. Differences in phenotype between genotypes were tested for statistical significance by Kruskal-Wallis/Mann-Whitney *U* (onset) or Chi-Square test (colon involvement/fistulae and stricture development). [a]$P \leq 0.03$ *vs* 5T6T and 6T6T combined, [c]$P = 0.025$ *vs* TC and CC combined.
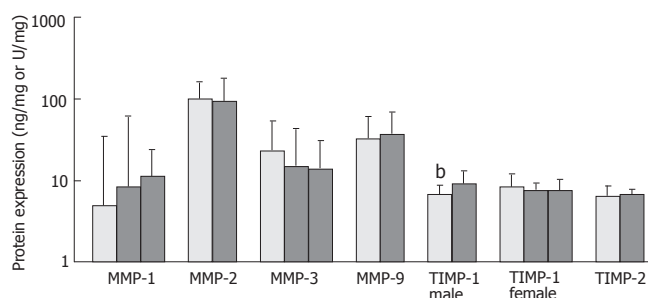

Figure 1 Protein expression in inflamed IBD tissue stratified to genotype. Values represent the median + 75th percentile and are in ng/mg (MMP-2, -9, TIMP-1, -2) or in arbitrary units/mg (MMP-1, -3). From left to right; MMP-1: 1G1G, 1G2G, 2G2G (*n* = 6, 17, 5); MMP-2: CC, CT + TT (*n* = 99, 77); MMP-3: 5T5T, 5T6T, 6T6T (*n* = 21, 33, 16); MMP-9: CC, CT + TT (*n* = 129, 46); TIMP-1 male: T, C (*n* = 47, 24); TIMP-1 female: TT, TC, CC (*n* = 42, 37, 26); TIMP-2: GG, GA + AA (*n* = 135, 41). [b]$P < 0.01$, Mann-Whitney *U* test.

developed peri-anal, entero-entero or entero-cutaneous fistulae during follow-up. Female patients with the TT genotype at +372 SNP of TIMP-1 appeared less prone to develop fistulae ($P = 0.08$), and when the TT group was compared with the combined TC and CC group, this result was statistically significant ($P = 0.025$).

Effect of SNPs on protein expression

Male IBD patients carrying the T allele at SNP +372 expressed lower levels of TIMP-1 in inflamed tissue compared to those carrying the C allele, $P = 0.009$ (Figure 1), with similar MPO levels in both groups [median 24.2 (range 9.1-80.4) *vs* 28.6 (2.5-75.9) U/g, $P = 0.194$]. In male CD patients a similar pattern in TIMP-1 expression was observed [6.8 (1.7-18.6) *vs* 9.2 (1.8-19.9) ng/mg TIMP-1, *n* = 46 *vs* 19, T *vs* C allele, respectively], although

not longer statistically significant ($P = 0.065$). However, female IBD or CD patients carrying the TT, TC or CC genotype expressed similar levels of TIMP-1 in inflamed tissue. The respective protein expression was not affected by genotype at other MMP and TIMP SNPs in inflamed intestinal tissue. In non-inflamed IBD and control CRC tissue, no differences in protein levels were observed between patients stratified to genotype. Finally, allelic composition at TNF-α-308 G/A was not associated with higher or lower levels of MMPs or TIMPs in inflamed and non-inflamed IBD or control tissue.

DISCUSSION

In this study we found increased susceptibility to CD in men and women carrying the T and TT genotype, respectively, at TIMP-1 SNP +372. The X chromosome region p11.3-p11.23 might thus represent a novel linkage marker in IBD, extending the results obtained in previous genome-wide linkage studies[14,34]. Women with this genotype also appear less prone to the development of fistulae. The direct or indirect involvement of the X-chromosome in CD etiopathogenesis is further corroborated by a higher incidence of CD in women compared to men[35], the association of CD with X-linked Turner's syndrome[8] and the higher incidence of extra-intestinal complications and surgery recurrence rates in female compared to male CD patients[36]. Importantly, in men the T allele at SNP +372 was accompanied with a lower TIMP-1 protein expression in inflamed tissue. The lower TIMP-1 protein levels relative to MMP in susceptible individuals might shift the balance to a more proteolytic mucosal Crohn's disease phenotype. The TIMP-1 SNP might also be linked to other markers on

the X-chromosome increasing CD susceptibility and conferring protection against fistulae pathogenesis thus explaining the observed results in women. We observed no association between allelic composition at MMP-3 SNP-1613 and susceptibility to IBD. Our findings in UC confirm previous publications on primary sclerosing cholangitis and UC[22,37], but those on CD are different from the results obtained by the group of Pender et al[38], who noted increased susceptibility to sporadic, but not familial CD in individuals carrying the 5T allele. These contrasting results might arise from a different proportion of sporadic versus familial cases in our study. We also found a decreased chance of colonic involvement at first endoscopic/radiologic examination and a higher incidence of stenotic complications in patients carrying the 5T5T MMP-3 genotype at SNP-1613. Previously, over transmission of the 5T allele was associated with ileal localisation and stenosis in CD CARD15 mutation carriers[38] and the group of Warnaar et al[39] reported increased levels of MMP-3 in stenotic and pre-stenotic resected CD ileum, pointing to an MMP-3 mediated altered clinical course of CD patients by an, as yet, unidentified mechanism. The 5T5T genotype was reported to both increase[40,41] and decrease[42] MMP-3 protein expression, but in our study patients stratified according to MMP-3 genotype expressed similar MMP-3 total activity. Previously, the A allele at TNF-α SNP-308 was reported to increase susceptibility to UC[43], CD[44] and the incidence of fistulae in CD[45], possibly mediated by an increased promoter activity[46,47]. In contrast, we found no effect of allelic composition at this SNP on disease risk and phenotype, in line with other reports[48,49], adding further complexity to this matter. As mentioned before, the patient populations might differ dependent on the genetic (ethnic) background, thus explaining the contrasting results. We could not demonstrate an association of MMP-1, -2, -9 and TIMP-2 SNPs with disease susceptibility or clinical course of disease, in line with previous (genome-wide) linkage reports[11-13,37,50]. As other studies have clearly shown the involvement of these proteins in IBD pathology[51,52], it seems that they primarily function as mediators/effectors instead of initiators during IBD etiopathogenesis. However, the regulation of these proteins by immuno-suppressive medication, such as infliximab, might be dependent on the allelic composition at the SNPs examined, as previously shown by ex vivo explant studies from our group[32]. In principle, enhanced MMP expression might also be associated with SNPs in other genes, for instance with those encoding cytokines regulating MMP expression, e.g., IL-1β[44] and TNF-α. Dependent on the presence of relevant cis-acting elements in the promoter sequence, especially MMP-1 and MMP-9 would be affected[53,54], but we found no effect.

In summary, several studies reported associations between SNPs in diverse genes and IBD[16,48,55-59]. We have focused on the SNPs in genes coding for matrix remodeling proteins, i.e., MMPs and TIMPs, and believe the T allele at SNP +372 T/C in TIMP-1 might be involved in CD susceptibility in both sexes and in men by down-regulating TIMP-1 expression, while the 5T5T genotype at MMP-3-1613 might protect for colonic disease

localization but also confers a major risk to stenotic complications. These findings reinforce the potential role of MMP and TIMPs in IBD and should be confirmed in larger prospective follow-up studies.

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