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Associations of distinct variants of the intestinal mucin gene *MUC3A* with ulcerative colitis and Crohn's disease

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Abstract Ulcerative colitis (UC) and Crohn's disease (CD), the major forms of inflammatory bowel diseases (IBDs), are multifactorial disorders of unknown etiology. We reported a possible association of rare variable number of tandem repeat (VNTR) alleles of the "*MUC3*" gene with a susceptibility to UC. However, an entire structure of "*MUC3*" is still unknown because the long stretches of tandem repeats in this "gene" make its cloning extraordinarily difficult. In this study, we report evidence that "*MUC3*" consists of two genes, *MUC3A* and *MUC3B*, both of which encode membrane-bound mucins with two epidermal growth factor-like motifs, and we describe the complete 3'-terminal structures of these two genes. We have also analyzed the single nucleotide polymorphisms (SNPs) in the exonic sequences of the 3' portions of these two genes to investigate whether sequence variations in these regions can cause person-to-person differences in the susceptibility to IBDs, and report here that non-synonymous SNPs of *MUC3A*, involving a tyrosine residue with a proposed role in cell signaling, may confer genetic predisposition to CD ($P = 0.0132$). Our findings suggest that variants of *MUC3A* may be involved in the occurrence of UC and CD in distinct manners.

Key words *MUC3A* · *MUC3B* · Ulcerative colitis · Crohn's disease · VNTR · SNP

Introduction

Ulcerative colitis (UC) and Crohn's disease (CD) are the major forms of inflammatory bowel diseases (IBDs). The etiology remains unknown, but it is currently accepted from the familial aggregation of IBD patients (Orholm et al. 1991) and from the increased yet incomplete concordance rates in monozygotic versus dizygotic twins (Tysk et al. 1988) that both environmental and genetic factors interact in the occurrence of these disorders. As for genetic factors, linkage analysis for susceptibility genes in IBDs involving 186 affected sibling pairs showed evidence for linkage to markers *D12S83*, *D7S669*, and *D3S1573* with logarithm of odds (lod) scores of 5.47, 3.08, and 2.69, respectively (Satsangi et al. 1996). The fact that the marker *D7S669* is located near the human intestinal mucin gene "*MUC3*" and previous reports that indicated a possible mucin abnormality in IBD patients (Buisine et al. 1999; Tysk et al. 1991) prompted us to examine a correlation between "*MUC3*" and IBDs. Genotyping of variable number of tandem repeat (VNTR) in the "*MUC3*" gene implied that rare alleles of "*MUC3*", which have an unusual number of 51-bp repeat units, are associated with the risk of UC (Kyo et al. 1999).

Mucins are large and heterogeneous glycoproteins subdivided into membrane-bound and soluble forms. They have important roles not only in the protection of epithelium, but in the adhesion and growth of epithelial cells, signal transduction, and modulation of immune system (Agrawal et al. 1998a; Agrawal et al. 1998b; Carraway et al. 1999). Each of the known mucin genes contains in its coding region a long stretch of repeated sequences. "*MUC3*" contains two separate regions of 51-bp tandem repeats, each covering several kilobases, and in one of their upstream regions lies a 177-bp repeat with the intervention of 810bp of a semi-unique coding region (van Klinken et al. 1997), and in the other lies a 1125-bp repeat without intervention of any other exon or intron sequences (Gum et al. 1997), and both repeats also cover several kilobases. The long stretches of tandem repeats of "*MUC3*" make its cloning extraordinarily difficult, and the entire structure of

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"*MUC3*" is still unknown, although 10 years have passed since the sequences of short partial cDNA clones of "*MUC3*" were first reported (Gum et al. 1990).

The sequence of the 3' region of "*MUC3*" was once reported, which indicated that "*MUC3*" was a secretory mucin with one epidermal growth factor (EGF)-like domain (Gum et al. 1997). We could not explain the dominant effect observed in the association study we reported recently (Kyo et al. 1999), because human *MUC3* was considered to be a secretory mucin and mucin protein encoded by a common allele would compensate for the function of that encoded by a rare allele. Furthermore, it has been unknown whether the 51-bp repetitive regions lie in a single gene, making an internal duplication, or whether they lie in two tandemly arrayed genes (Gum et al. 1997). We reanalyzed the 3' terminal structure of "*MUC3*", and here we demonstrate that "*MUC3*" consists of two genes, *MUC3A* and *MUC3B*, each of which possesses two EGF-like domains and a putative transmembrane region, and we again discuss the implication of the association of rare alleles of *MUC3A* with UC.

Single nucleotide polymorphisms (SNPs) have a great potential for application to association studies with diseases and pharmacogenetic traits (Chakravarti 1999; Housman and Ledley 1998), and SNPs in coding regions (cSNPs), particularly those that alter amino acid sequences of the encoded proteins, are most likely to influence the protein function and thus be involved in complex human diseases (Chakravarti 1999; Cargill et al. 1999). We identified and analyzed SNPs in the exonic sequences of the 3' regions of these two genes for the association with IBDs, and have found that non-synonymous SNPs of *MUC3A* involving a tyrosine residue with a proposed role in cell signaling may be involved in the person-to-person differences in the susceptibility to CD. Thus, we report here that distinct variants of *MUC3A* may be involved in the occurrence of UC and CD.

Materials and methods

Southern blot analysis

Five micrograms of genomic DNA were digested to completion with restriction enzyme *PvuII* (Toyobo, Tokyo, Japan), run in 0.5% agarose gel (13cm long) at 25 V for 48h and transferred to a nylon membrane, as described previously (Kyo et al. 1999). Blots were probed with a clone composed of a 51-bp repeat (51R), a 498-bp clone corresponding to a part of 1125-bp repeat unit located between *PvuII*-restriction site and 51-bp repeat (1125D), a 796-bp clone corresponding to a semi-unique region located between the 177-bp repeat and the 51-bp repeat (SUR), or three distinct parts of the 3' region of "*MUC3*", the sequence of which Gum et al. (1997) reported previously (3TR-1, 3TR-2, or 3TR-3; Fig. 1b). Clone 51R was prepared as described (Kyo et al. 1999), and clone 1125D was prepared by PCR amplification, using primers 1125DF and

1125DR (Table 1); the PCR reaction consisted of one cycle of denaturation at 95°C for 5 min, 35 cycles of denaturation at 94°C for 30s, annealing at 57°C for 30s, and extension at 72°C for 30s. Clone SUR was also prepared likewise, using primers SUR-F and SUR-R (Table 1); the PCR regime included one cycle of 95°C for 5 min, 35 cycles of 94°C for 30s, 57°C for 30s, and 72°C for 1 min. Clones 3TR-1 to -3 were prepared by PCR, using primer sets 3TR-1F/3TR-1R, 3TR-2F/3TR-2R, and 3TR-3F/3TR-3R (Table 1), respectively, which were constructed on the basis of the reported sequence (Gum et al. 1997); the PCR regime included one cycle of 95°C for 5 min, 35 cycles of 94°C for 30s, 57°C for 30s for 3TR-1 and 3TR-3 (55°C for 30s for 3TR-2), and 72°C for 1 min for 3TR-1 (for 30s for 3TR-2 and 3TR-3). Each of the PCR reaction mixtures was prepared in 30μl of reaction volume containing 20ng of genomic DNA, 3μl of 10 × PCR buffer [166mM (NH₄)₂SO₄, 670mM Tris (pH8.8), 20mM MgCl₂, 100mM β-MeEtOH, 67μM ethylenediamine tetraacetic acid (EDTA)], 1.5U of Ex Taq polymerase (TaKaRa, Tokyo, Japan), 20pmol of each primer, 1.5mM dNTPs, 4.7mM MgCl₂, and 10% dimethylsulfoxide (DMSO). These clones were confirmed by sequencing and were labeled by the random priming method, using a Megaprime DNA labeling system (Amersham, Buckinghamshire, UK) according to the manufacturer's instructions. The hybridization and washing of the blots were carried out as described (Kyo et al. 1999).

Sequence analysis

As demonstrated in Fig. 1a, two sets of bands were observed in the Southern analysis when *PvuII*-digested genomic DNA fragments were hybridized with the clone 51R as a probe. The larger set of hybridizing bands was 20 to 40kb in size, and the smaller set was 10 to 15kb. These bands were also detected with clones 3TR-1 to -3, suggesting that the 3' regions of these two genes share high homology with each other. We hypothesized, because of the reason, noted in the "Discussion" section, that these sets of hybridizing bands were derived from distinct genes, and we designated, as recommended by the Human Gene Nomenclature Committee, the genes which contained smaller and larger sets of hybridizing bands as *MUC3A* and *MUC3B* genes, respectively.

PvuII-digested genomic DNA fragments were separated in the 0.5% agarose gel (25cm long), run at 40V for 72h, and fragments from 20 to 40kb in size and those from 10 to 15kb were cut out and purified, and were utilized as templates for PCR amplifications, using common primers 3TR-1F and 3TR-3R (1A and 1B in Fig. 1b; Table 1). The PCR reaction mixtures were prepared as mentioned above, and the reactions consisted of one cycle of 95°C for 5 min followed by 32 cycles of 94°C for 30s, 57°C for 30s, and 72°C for 3 min. Each of the amplified products was directly sequenced on both strands with an ABI 377 Autosequencer (Perkin-Elmer, Norwalk, CT, USA). A genomic DNA sample from the same person was utilized for the following analysis of the genomic structures of these two genes.

Fig. 1a–d. Genomic structures of the 3' regions and tissue expressions of *MUC3A* and *B*. **a** Autoradiographs of Southern blots showing hybridization with clones 51R, 1125D, SUR, 3TR-1, 3TR-2, or 3TR-3. *PvuII*-digested genomic DNA fragments (5 μg) were size-fractionated on a 0.5% agarose gel and transferred to a nylon membrane prior to hybridization. The positions of clones 3TR-1, 3TR-2, and 3TR-3 are demonstrated in **b**. **b** Upper molecule; schematic representation of the genomic structure once reported as the 3' region of “*MUC3*” (Gum et al. 1997). The sequences of CEX.III (see below) were included in an intronic sequence. The positions of clones 3TR-1, 3TR-2, and 3TR-3, which were used as probes in the Southern analysis, are indicated with bars below the figure. The *PvuII*-restriction site is shown with an arrow. **b** Middle and lower molecules; schematic overview of the genomic

structures of the 3' region of *MUC3A* (middle molecule) and *MUC3B* (lower molecule). Exons and introns are indicated by boxes and lines, respectively. Boxes with vertical hatching indicate the 51-bp repeat. Exons in the 3' region of each gene are numbered consecutively as CEX. I to XI above the boxes. Introns are named below the lines in capital letters. The positions of clones obtained by polymerase chain reaction (PCR) amplifications in the analyses of genomic structures (1A–5A for *MUC3A* and 1B–5B for *MUC3B*) and in the identification of single nucleotide polymorphisms (SNPs) (IA–IVA for *MUC3A* and IB–IVB for *MUC3B*) are indicated with bars below the figures. **c, d** Northern blot analyses of *MUC3A* (**c**) and *MUC3B* (**d**) genes. RNA blots containing 2 μg of poly A⁺ RNA from each of 16 human tissues were hybridized with clones 1125D for *MUC3A* or 117R for *MUC3B*

Table 1. Sequence of PCR primers

Southern analysis			
1125DF	GAATCCACCCCATCACCTAC	1125DR	GGTGAATAGGTGGTTCTGG
SUR-F	GTACCACACCTGTTACGAGG	SUR-R	GATGCAGAAGTAGTGCAGCC
3TR-1F	TCAACATCTACTCCACAGTC	3TR-1R	GAAGAAGGAACAGAATGAATGG
3TR-2F	TCTGTTCCCTCCTTCCTC	3TR-2R	GGAGAGGAGCATGACACAG
3TR-3F	CTCCGTCTCACCCCTTAGG	3TR-3R	CTGATCTTGAGAAAATTAACATAG
3'RACE			
1) <i>MUC3A</i>			
A-1	CTGAGCAACAGTTCTGTGATCCCCCT	AN-1	CGCAGGACAACCTCGCATCACTTCTCA
A-2	CGGGCTATGAAGAGTTCTACTTCCCCT	AN-2	GCTGTGTCACCAAATGCACGTCTGGG
A-3	GAATCTTGGGCAAGTCAGTAACGAGCC	AN-3	GCATTCTGTATGATAGCTCACGCCG
A-4	CACCCTGCTGCCCAATTCTTTATTC	AN-4	TTTCTCTCACCCCTGGAGCCCTGC
2) <i>MUC3B</i>			
B-1	CTGAGCAACAACCTCTGTGATCCCCAC	BN-1	CGCAGGACAACCTCCCATCACTTCTTG
B-2	CGGGCTATGAAGAGTTCTACTTCCCTC	BN-2	GCTGTGTCACCAAATGCACGTCTGGG
B-4	CCAGCCACCGGATTCTCCATTAC	BN-4	TTTCTCTCACCCCTGGAGCCCCGT
3) Linearity check			
AF1	AGACCTGGCTGAGCAACAGT	BF1	AGACCTGGCTGAGCAACAAC
RPR-1	CAGGGTCATGGGGGGAAC	RPR-2	ACGGGGTGAGGGGGGAATT
RT-PCR analysis of splicing variants			
ABR1	ACTGAGGACGAGGTCATCTC		
Analysis of genomic structures			
AF2	TGGAGCCTCCACAGCAAG	BF2	CCATCTTCCAAGCCAGCAAG
ABR2	AAGGTGAAGCCCTGCATGTC	ABF1	TGGAGATCCTGTCCCTGAG
AR1	TAGCCCCACCCTTGGCAC	BR1	CTAGCCCCACTCTTGATGG
AF3	CCTCCAAGGACCCATATGTT	BF3	CTCCAAGGACCCATGTGCC
AR2	GTCGATGGCGTTGTCCACC	BR2	GTCGATGGCGTTGTCCACG
AF4	TCACCAAATGCACGTCTGGG	BF4	CACCAAATGCACGTCTGGG
ABR3	TCCTGTCCCCAAAGCCAATC		
Northern analysis			
117RF	TCTCAGTACCACGCCGGTG	177RR	AGGCATAATGGTTAATGGAGTG
SNP identification			
1) <i>MUC3A</i>			
AF5	CATCTACTCCACAGTCAGCA	ABR4	CCCACAGAGGGGATAAGTCTG
AF6	CTCAGCCTCCTGAGGCATA	ABR5	GGGAGTGATTCTTATGTACC
AR3	GGCGGAGCATGGGGCAG	AF7	GTTCTAGGGTGGATTCCCAG
AR4	CAATCTTCCCCGCCAAGGC		
2) <i>MUC3B</i>			
BF5	GGGTGACTTCCACACCTTC	BF6	CTCAGCCTCCCAAGGCACT
BR3	GGCGGCGCGTGAGGCTA	BF7	TTCTACGGTGGATTCCGGC
BR4	CAATCTTCCCCGCCAAGGA		
3) Association analysis			
AF8	CCTTCAAACAACCTCCTCTACT	ABR6	TTATAGTTCCTGGACAGGGTG
AF9	CTGCAACTACCAGCACTTCTT	AR5	GAACTGGAAGCATTGGGTGA
ABR7	CCAGAGGTACCACAGGGCT	ABF2	CGGAAATGGAATCCTCCTCG

PCR, Polymerase chain reaction; RACE, rapid amplification of cDNA ends; RT, reverse transcription; SNP, single nucleotide polymorphism

3' Rapid amplification of cDNA ends (RACE)

The sequence differences observed in the two PCR-amplified products mentioned above enabled us to construct RACE primers specific to each gene. Four rounds of RACE reactions were conducted for each of the *MUC3A* and *MUC3B* genes to reach the poly (A) tail, using a Marathon cDNA amplification kit and Marathon-Ready cDNA from adult small intestine (both from Clontech, Palo Alto, CA, USA). Gene specific primers A-1 to -4 for the *MUC3A* sequence and B-1, -2, and -4 for the *MUC3B* sequence were used for primary amplifications, and AN-1 to -4 and BN-1, -2 and -4 were used as gene-specific nested primers (Table 1). The primers used for the first round of RACE reactions were constructed on CEX. I (Fig. 1b). In the third round of RACE amplifications, common primers A-3 and AN-3 were used for both *MUC3A* and *MUC3B*, because no sequence difference was found between the second round of amplified products of *MUC3A*

and *MUC3B*. Because two distinct sequences with a high degree of similarity were obtained in the third round of amplifications, each linearity was checked by PCR, using primers AF1 for *MUC3A* and BF1 for *MUC3B*, and primers RPR-1 and RPR-2, which were constructed on the amplified products of the third-round reactions (Table 1).

For the RACE reactions, we used a Perkin-Elmer Thermal Cycler Gene Amp PCR System 9700, and each of the primary reactions consisted of denaturation at 94°C for 30s, followed by five cycles of denaturation at 94°C for 10s, annealing and extension at 72°C for 4min, with five subsequent cycles of 94°C for 10s and 70°C for 4min, followed by 25 remaining cycles of 94°C for 10s and 68°C for 4min. Each of the nested-PCR reactions consisted of one cycle of 94°C for 30s followed by five cycles of 94°C for 10s and 72°C for 4min, with five subsequent cycles of 94°C for 10s and 70°C for 4min, followed by 20 remaining cycles of 94°C for 10s and 68°C for 4min.

After the amplified products were electrophoresed on agarose gels, bands were cut out, cloned into pBluescript II SK(–) and sequenced on both strands with an ABI 377 Autosequencer (Perkin-Elmer). At least five clones for each were sequenced for comparison between the *MUC3A* and *MUC3B* sequences.

Reverse transcription (RT)-PCR analyses of splicing variants

One microgram of poly A⁺ RNA isolated from small intestine (Clontech) was reverse-transcribed, using an oligo-dT17 primer (Boehringer Mannheim, Mannheim, Germany) and Superscript II RNase H[–] reverse transcriptase (GIBCO BRL, Rockville, MD, USA). The resultant cDNA product was amplified by PCR, using primer sets AF1/ABR1 for *MUC3A* and BF1/ABR1 for *MUC3B* (Table 1). These primers were constructed on CEX. I and CEX. XI of *MUC3A* and *MUC3B* (Fig. 2b,c). The PCR reaction mixtures were prepared as mentioned above, and the PCR regime included one cycle of 95°C for 5 min, then 32 cycles of 94°C for 30s, 55°C for 30s, and 72°C for 2 min. The PCR products were separated on an agarose gel and all visible bands were cut out and cloned into pBluescript II SK(–), and sequenced on both strands with an ABI 377 Autosequencer (Perkin-Elmer).

Analyses of genomic structures of the 3' regions of *MUC3A* and *MUC3B* genes

Genomic structures of the 3' regions of the *MUC3A* and *MUC3B* genes were analyzed by PCR amplifications conducted in five overlapping regions (Fig. 1b). Region 1 of each gene (1A and 1B) was amplified by PCR using primers 3TR-1F and 3TR-3R (Table 1), as mentioned above. For the amplifications of regions 2 to 5, primer sets of AF2/ABR2, ABF1/AR1, AF3/AR2, AF4/ABR3 for *MUC3A* (2A to 5A), and BF2/ABR2, ABF1/BR1, BF3/BR2, and BF4/ABR3 for *MUC3B* (2B to 5B) were used (Table 1). Each of the reactions consisted of denaturation at 95°C for 5 min followed by 32 cycles of denaturation at 94°C for 30s, annealing at 55°C for 30s for regions 3A, 3B, and 4A or at 57°C for 30s for all others, and extension at 72°C for 2 min. The amplified products were cloned into pBluescript II SK(–), and at least five clones were sequenced for each, in both directions, with the ABI 377 Autosequencer (Perkin-Elmer). The position, size, and sequence of the introns were confirmed by comparison of PCR-amplified products of genomic DNA with products of RT-PCR mentioned above.

Comparison of sequences to databases and examination of amino acid sequences for potential structural domains were performed using the BLAST, FASTA, and MOTIF programs accessed through the Human Genome Center, Institute of Medical Science, University of Tokyo (<http://www.genome.ad.jp/>), and ExPASy sequence analysis programs (<http://www.expasy.ch/>). The genomic sequences of the 3' regions have been submitted to the GenBank database with accession numbers AB038782 for *MUC3A* and AB038781 for *MUC3B*. The cDNA sequences of the major

transcripts have also been submitted, with accession numbers AB038784 for *MUC3A* and AB038783 for *MUC3B*.

Northern blotting analysis

Multiple-tissue Northern blots (Clontech) containing 2 µg of poly A⁺ RNAs from heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon, and leukocyte were used for analysis. Clone 1125D and a 532-bp clone 177R, composed of a 177-bp repeat, were used to investigate the expression of *MUC3A* and *MUC3B*. Clone 177R was prepared by cloning into pBluescript II SK(–) the PCR products amplified with primers 177RF and 177RR (Table 1). The PCR utilized genomic DNA as a template, and consisted of 95°C for 5 min, followed by 32 cycles of 94°C for 30s, 57°C for 30s, and 72°C for 30s. Probes were labeled with [α -³²P] dCTP, using the Megaprime DNA labeling system (Amersham). Hybridization and washing of the blots were performed as per the manufacturer's instructions.

Identification and association analysis of SNPs

To investigate whether some sequence variations contribute to the susceptibility to IBDs, we screened genomic DNA samples isolated from 30 patients with UC and 30 with CD, and surveyed the exon sequences of the 3' regions of *MUC3A* and *MUC3B*. These sequences were amplified by PCR, using gene-specific primer sets AF5/ABR4 (for IA), AF6/ABR5 (IIA), AF3/AR3 (IIIA), and AF7/AR4 (IVA) for *MUC3A*, and BF5/ABR4 (IB), BF6/ABR5 (IIB), BF3/BR3 (IIIB) and BF7/BR4 (IVB) for *MUC3B* (Fig. 1b, Table 1). The reaction consisted of 95°C for 5 min followed by 35 cycles of 94°C for 30s and 57°C for 30s for IVA and IIB (55°C for 30s for all others) and 72°C for 1 min for IIA and IIB (72°C for 2 min for all others). The amplified products were directly sequenced by mixing three samples in one with an ABI 3700 Autosequencer (Perkin-Elmer) in both directions, and SNPs were identified using a combination of the PolyPhred program (Nickerson et al. 1997) followed by visual inspection.

For each of the identified SNPs, 72 sporadic cases of UC and 70 unrelated patients with CD, as well as ethnically matched controls, were genotyped by PCR amplifications, using primers specific to each gene, followed by allele-specific oligonucleotide hybridization (ASO), except for nucleotide (nt) 3337 to 3398 of *MUC3A* and nt 155 to 270 and 3141 to 3469 of *MUC3B* (Table 2), where genotyping was done by sequencing because the close proximity between SNP sites makes hybridization difficult. Details of the UC patients and controls were described previously (Kyo et al. 1999). The total of 70 CD patients consisted of 44 sporadic CD cases, with the remaining 26 patients being the younger brothers or sisters of CD-affected siblings. Sporadic CD cases were ascertained through the Department of Surgical Oncology, the University of Tokyo, or through the Second Department of Surgery, School of Medicine, Hamamatsu University. CD-Affected siblings were

Fig. 2a-c. Reverse transcription-polymerase chain reaction (RT-PCR) analyses of splicing variants. **a** RT-PCR amplifications of transcripts of *MUC3A* and *MUC3B*. Gene-specific primers used for the amplifications were constructed on CEX. I and XI of each gene. **b, c** Schemes depicting various transcripts of *MUC3A* (**b**) and *MUC3B* (**c**). White and gray boxes indicate coding and non-coding regions, respectively. Introns are represented by lines. Optional exons are indicated with "elbowed" lines. Epidermal growth factor (EGF)-like domains and a transmembrane domain are marked with blue and red boxes, respectively. The sequence of CEX. V" of *MUC3A* includes that of

CEX. V and 172 bp of the 3'-side sequence of intron E. The sequence of CEX. XII corresponds to that of nucleotide (nt) 4048 to nt 4140 in AB038782. **d** Similarity of the deduced amino acid sequences between the carboxyl termini of the major transcripts of *MUC3A* and *MUC3B*. Both sequences begin at the amino acids immediately downstream of the 51-bp repeats. Dark shading indicates identical amino acids, and hyphen indicates a gap inserted to optimize the alignment. The EGF-like motifs (EGF1 and EGF2) and the putative transmembrane regions (TM) are underlined. The potential N-glycosylation sites are indicated in *italics*

Table 3. ASO probes for single nucleotide polymorphisms

Allele	Sequence (5' to 3')	Discriminatory temperature (°C)
771C	TCCAGTGTCTTCCACTACC	66
771T	TCCAGTGTTTCCACTACC	64
1005T	CCTGCCTCTCCCACTGA	64
1005C	CCTGCCTCCCCCACTGA	66
2021C	CCAGGATGCGAACAGCT	62
2021T	CCAGGATGTGAACAGCT	60
2205T	CACGTCTGGGGTGGAC	66
2205G	CACGTCTGGGGTGGAC	68
2557A	AATTTCAAATGTGGCCTTG	64
2557C	AATTTCCATGTGGCCTTG	64
2557T	AATTTCTATGTGGCCTTG	64

ASO, allele-specific oligonucleotide

identified by a nationwide survey, and medical records were reviewed for all affected siblings to confirm the diagnosis. Table 3 shows the sequences of oligonucleotide probes utilized in the genotyping at five polymorphic sites (nt 771, 1005, 2021, 2205, and 2557 of *MUC3A*; Table 2) where we found associations with familial CD. PCR amplifications for the genotyping were done by primer sets AF8/ABR6, AF9/BR5, AF3/ABR7, ABF2/AR3 and AF7/ABR1, respectively. The PCR conditions were 95°C for 5 min, followed by 35 cycles of 94°C for 30s, 57°C for 30s for genotyping at nt 771 and 2557 (55°C for 30s for all others), and 72°C for 1 min for nt 2557 (for 30s for all others). In ASO hybridization analyses PCR-amplified DNAs were spotted onto Biotodyne A nylon membranes (Pall corporation, East Hills, NY, USA) and UV-crosslinked. Oligonucleotide probes were end-labeled with γ -32P-dATP, using T4 polynucleotide kinase (Toyobo; Sambrook et al. 1989), and hybridized to the membranes at 42°C–50°C in a solution containing $6 \times$ standard saline citrate (SSC), 0.5% SDS, 100 μ g/ml salmon sperm DNA, $2 \times$ Denhardt's solution, and 50mM Tris-HCl (pH 7.4). High-stringency washes were done in $6 \times$ SSC at the discriminatory temperatures specified for each ASO (Table 3). Data values were analyzed using a χ^2 test.

Heterozygosity at each SNP site was estimated as $2(pq + qr + rp)$, where p, q, r indicate the proportion of each allele at each SNP site ($p + q + r = 1$; $r = 0$ except at nt 2557 of *MUC3A* and nt 2560 of *MUC3B*); for comparison of sequence diversity we estimated the nucleotide diversity as the average heterozygosity per base pair (π).

Results

Analyses of nucleotide and amino acid sequences of the 3' regions of *MUC3A* and *MUC3B* genes

As reported previously (Kyo et al. 1999), two distinct sets of hybridizing bands were recognized in the Southern analysis using the clone 51R as a probe (Fig. 1a). These bands were also detected with clones 3TR-1, 3TR-2, or 3TR-3, suggesting that the downstream regions of the two 51-bp repeats

share high homology with each other. However, only the smaller set of bands was detected with clone 1125D, and a quite different band, of approximately 16kb in size, was detected with clone SUR (Fig. 1a), indicating that the 1125-bp repeat belongs to *MUC3A* and the 177-bp repeat to *MUC3B*. Based on the expected high homology between the unique regions of these two genes, we amplified and analyzed these sequences, using *PvuII*-digested genomic DNA fragments of 10–15kb or of 20–40kb in size as PCR templates and common primers constructed on the basis of the reported sequence (Gum et al. 1997). The size of the amplified products was 2421 bp in *MUC3A* and 2463bp in *MUC3B*. The sequence of *MUC3A*, compared with that of *MUC3B*, had a three-base deletion of the nucleotides ACA in CEX. I and a 39-base deletion in intron B (Fig. 1b and Fig. 3a), and these deletions were observed irrespective of the alleles we reported (Kyo et al. 1999). The similarity of the nucleotide sequences of the two amplified products was 94.8% and that of the exonic sequences reached 95.8%, and the sequence differences between CEX. I of *MUC3A* and that of *MUC3B* enabled us to construct RACE primers specific to each gene. We conducted four rounds of RACE reactions for each of *MUC3A* and *MUC3B*; the compiled nucleotide sequences of *MUC3A* and *MUC3B* are demonstrated in Fig. 3. The analysis of splicing variants (Fig. 2) showed that the sequences obtained in RACE reactions were those of the major transcripts in both *MUC3A* and *MUC3B* and in these transcripts, the unique sequences downstream of the repetitive domains of *MUC3A* and *MUC3B* consist of 3808-bp and 3941-bp nucleotides that encode 877- and 878-residue peptides, followed by 3'-untranslated regions of 1177bp and 1307bp, respectively. The nucleotide and deduced amino acid sequences matched 94.1% and 94.9%, respectively, and each of the deduced amino acid sequences revealed a large extracellular domain (779 and 780 amino acids of *MUC3A* and *MUC3B*, respectively), a transmembrane domain of 23 amino acids, and a cytoplasmic tail of 75 amino acids (Fig. 2d). Comparison of the PCR-amplified genomic sequences with the RT-PCR products revealed 11 exons in the 3' regions of *MUC3A* and *MUC3B* spanning 8912bp and 9131bp of genomic DNA, respectively. The degree of sequence similarity between the two genomic sequences reached 91.3%. The sequence which was reported as that of 3' region of "*MUC3*" (Gum et al. 1997) corresponds to that of *MUC3A* in our study, although there were several errors (deletions, insertions, and substitutions) in the reported sequence. In that report, the sequence of CEX. III involved in introns and stop codon appeared in CEX. IV (Fig. 1b), which led to the conclusion that "*MUC3*" is a secretory mucin. Recently, Williams et al. (1999a) reanalyzed the cDNA structure of the 3' region of "*MUC3*", by conducting RT-PCR with primers constructed on the basis of the reported sequence, and they reported that "*MUC3*" encodes a transmembrane mucin. The sequence they reported is identical to the partial cDNA sequence of the 3' region of *MUC3A* in our study. Crawley et al. (1999) recently reported the genomic structure of the 3' region of "*MUC3*" (now *MUC3A*) correcting errors in the sequence they once reported. The sequence of *MUC3A* in

Fig. 3a-c. Similarity of exon sequences between the 3' regions of the major transcripts of *MUC3A* and *MUC3B*. The sequence begins at the nucleotides immediately downstream of the 51-bp repeats. *Dark shading* indicates identical nucleotides, and *hyphens* indicate gaps inserted to optimize the alignment. The EGF-like motifs (*EGF1* and *EGF2*)

and the putative transmembrane regions (*TM*) are *underlined*. The termination codons are marked with *asterisks* (Genbank accession number: AB038784 for *MUC3A* and AB038783 for *MUC3B*). The genomic sequences are also deposited with GenBank, accession nos, AB038782 for *MUC3A* and AB038781 for *MUC3B*

b **Fig. 3a–c.** Continued

our study still differed from theirs in some respects; there are four nucleotide substitutions in their sequence (A, T, C, G, rather than C, C, G, C at nt 1563, 2837, 2922, 2923 of *MUC3A*, respectively: Fig. 3), which we did not identify in the SNP analysis.

Although each of the repetitive regions of the *MUC3A* and *MUC3B* genes has the amino acid composition typical

of a mucin gene, the deduced amino acid sequences of CEX. I of *MUC3A* (and *MUC3B*) were also mucin-like, consisting of Thr 27.1% (25.5%), Ser 21.6% (22.2%), and Pro 11.0% (11.4%), accounting for 59.7% of 509 amino acids (59.0% of 510 amino acids), and two putative N-glycosylation sites were observed in CEX. I of each gene (Fig. 2d). The mucin-like domain was followed by two Cys-

MUC3A	CAGTTTCCTC	ACCTGCAAAA	CGGGTACAGC	ATTCTCTGTAT	GATAGCTCAC	GCCGTTGTTG	TGAAAACCA	ATAGACTTGG	2957	
MUC3B	CAGTTTCCTC	ACCTGCAAAA	CGGGTACAGC	ATTCTCTGTAT	GATAGCTCAC	GCCGTTGTTG	TGAAAACCA	ATAGACTTGG	2960	
MUC3A	TCAATTCTCG	GTCCTACTCT	GCCCTCCCGT	CTCAGCCCTC	GTGTTGCCAT	TGCCTCTCTC	GGATCCTCCA	ATCCTCACGT	3037	
MUC3B	TCAATTCTCG	GTCCTACTCT	GCCCTCCCGT	CTCAGCCCTC	GTGTTGCCAT	TGCCTCTCTC	GGATCCTCCA	ATCCTCACGT	3040	
MUC3A	CCTTCACCTG	GTCTCTGGCC	CTGGTTCTTA	TTTTCTCTCA	ATTCCCTACT	GCCTGTTTCT	TACTTTGAAC	CTGGAGGCAG	3117	
MUC3B	CCTTCACCTG	GTCTCTGGCC	CTGGTTCTTA	TTTTCTCTCA	ATTCCCTACT	GCCTGTTTCT	TACTTTGAAC	CTGGAGGCAG	3120	
MUC3A	CCTGCAGCCC	CATCCCATCT	CCTGCCCTCT	CCTGATCTAA	CTCCCTGCTG	CATCTCTTGG	TCTCATTCTT	TAGACGTCCT	3197	
MUC3B	CCTGCAGCCC	CATCCCATCT	CCTGCCCTCT	CCTGATCTAA	CTCCCTGCTG	CATCTCTTGG	TCTCATTCTT	TAGACGTCCT	3200	
MUC3A	CCCCTTTTGA	CCCCGTTTCT	TCATCCATCC	TGCACCCAG	TCCCCAGCC	CTAAATCCTC	CCTCCTCTCC	TCACATCCTG	3277	
MUC3B	CCCCTTTTGA	CCCCGTTTCT	TCATCCATCC	TGCACCCAG	TCCCCAGCC	CTAAATCCTC	CCTCCTCTCC	TCACATCCTG	3280	
MUC3A	GTCCCTAGCA	AGGTATAGAT	AGCCTCTGTG	TCTTAGGATA	CCCCGGGTGC	TGTTCCCTCG	GTACCCCTGT	TGCCCAGTTG	3357	
MUC3B	GTCCCTAGCA	AGGTATAGAT	AGCCTCTGTG	TCTTAGGATA	CCCCGGGTGC	TGTTCCCTCG	GTACCCCTGT	TGCCCAGTTG	3360	
MUC3A	CCCGTTTCTC	TTGCTCTCAT	TCCTTGATAT	CTTTCCCTTT	TTGAGCCCGT	CCATTTCATG	GTTCCTGCCC	CGACTCCCCC	3437	
MUC3B	CCCGTTTCTC	TTGCTCTCAT	TCCTTGATAT	CTTTCCCTTT	TTGAGCCCGT	CCATTTCATG	GTTCCTGCCC	CGACTCCCCC	3440	
MUC3A	AGCCCTAAAT	ACCCAGCTG	CTGTTCCTCC	CATCACCTTG	CTGGCCAATT	CTTTCTCTCA	TTCTTGTAT		3491	
MUC3B	AGCCCTAAAT	ACCCAGCTG	CTAATTCTCC	CTCACCTG	TTGGTCAATT	CTTTCTCTCA	TTCTTGTAT		3520	
MUC3A									3491	
MUC3B	CTTCTCCCC	TCTGAGCCTG	TCCATTATC	GGTGGTCTG	CCCCACTCC	CCCAGCCCTA	AATACCCAG	CTGCTGTTCC	3600	
MUC3A			ATTCTCCA		CCCCCTT	CTCTCACCC	TGGAGCCG	TGGGTGGGG	CAGGGCATGA	3546
MUC3B	TCCCCATCAC	CCAGCCACCG	GATTCTCCAT	TCA	CCCCCTT	CTCTCACCC	TGGAGCCG	TGGGTGGGG	CAGGGCATGA	3680
MUC3A	GTTCCTCCAGT	CCCCAAGGAA	AGGCAGCCCC	CTCAGTCTCC	CTCCTCCTCA	TTCCCTTCCA	TCTCCTCTCC	CTCTGCCTTT	3626	
MUC3B	GTTCCTCCAGT	CCCCAAGGAA	AGGCAGCCCC	CTCAGTCTCC	CTCCTCCTCA	TTCCCTTCCA	TCTCCTCTCC	CTCTGCCTTT	3760	
MUC3A	TAAACCCATC	CCCTCCGATT	CCCCTCCTCC	CCCCTCTCTC	CCTGGTGTCA	CCTGGATTCC	TGGAGTAAT	CTGAGCCCTT	3706	
MUC3B	TAAACCCATC	CCCTCCGATT	CCCCTCCTCC	CCCCTCTCTC	CCTGGTGTCA	ACTCGATTCC	TGGAGTAAT	CTGAGCCCTT	3839	
MUC3A	GAAATCCTCA	GTCTCCTTGG	CGGGGAAGAT	TGGCTTTGGG	GACAGGAAGT	CGGCACATCT	CCAGGTCTTC	ATGTGCACAA	3786	
MUC3B	GAAATCCTCA	GTCTCCTTGG	CGGGGAAGAT	TGGCTTTGGG	GACAGGAAGT	CGGCACATCT	CCAGGTCTTC	ATGTGCACAA	3919	
MUC3A	TATAGAGTTT	ATTGTAAAAA	GC						3808	
MUC3B	TATAGAGTTT	ATTGTAAAAA	GC						3941	

Fig. 3a–c. Continued

rich, epidermal growth factor (EGF)-like domains separated by 168 amino acids containing additional five putative N-glycosylation sites (Fig. 2d). The first EGF-like domain (EGF1) consists of 40 amino acids (residue 523–562 for *MUC3A* and 524–563 for *MUC3B*, Fig. 2d), and the second domain (EGF2) consists of 42 amino acids (residue 731–772 for *MUC3A* and 732–773 for *MUC3B*, Fig. 2d) which was immediately followed by a transmembrane domain.

RT-PCR analyses of splicing variants (Fig. 2) revealed several variants with or without a transmembrane region in each of *MUC3A* and *MUC3B*, indicating that the secretory forms of *MUC3A* and *MUC3B* mucin proteins are produced by alternative splicing rather than by proteolytic cleavage, as observed in *MUC1* and *MUC4* (Ligtenberg et al. 1992; Moniaux et al. 1999). Each gene has a variety of cryptic splicing sites, which resulted in novel exons including intronic sequences. All transcripts shared EGF1, and those with a transmembrane region exclusively shared EGF2.

Tissue expressions of *MUC3A* and *MUC3B*

Northern blots, using clone 177R as a probe, showed expression of *MUC3B* exclusively in the small and large intestines (Fig. 1d) whereas that of *MUC3A*, analyzed with clone 1125D, was observed at the highest level not only in the intestines but also in heart, liver, and thymus, with lower levels in prostate and pancreas (Fig. 1c). As described previously for mucin RNAs (Debailleul et al. 1998), these messages were highly polydispersed because of shearing of the extremely large transcripts.

Identification and association analysis of SNPs

We screened for SNPs in 3697 and 3766bp of exonic sequences of the 3' regions of the *MUC3A* and *MUC3B* genes, which consisted of 2609-bp coding and 1088-bp noncoding sequences for *MUC3A*, and of 2548-bp coding

and 1218-bp non-coding sequences for *MUC3B* (Table 4). We identified 11 SNPs and 13 rare variants in *MUC3A*, and 18 SNPs in *MUC3B*, corresponding to $\pi = 11.2 \times 10^{-4}$ for *MUC3A* and 15.5×10^{-4} for *MUC3B* (Table 4). These SNPs and rare variants are summarized in Table 2, with the allelic frequencies observed in the control population. Despite the equal number of samples screened, rare variants were identified only in *MUC3A*. Among these variants, 7 variants, of 199C → G (P67A), 380T → C (I127T), 666G → A (T222T), 751G → T (A251S), 903T → C (S301S), 914C → G (A305G), and 1381A → G (S461G), were observed in a single patient with CD, and were also identified in only one control sample (frequency of 0.26% in 384 control alleles). The results indicate that these seven mutations occurred at the same time. Likewise, three mutations in the 3' non-coding region (3388C → T, 3390T → C and 3398T → C) were identified in a single UC patient and also in a single control sample. Individuals carrying rare variants of *MUC3A* were identified more often in IBD patients than in controls (8.3% vs 2.1%; Fisher's exact *P* value = 0.0374).

cSNPs, particularly those that alter amino acid sequences, are most likely to influence the protein function and, thus, to be involved in complex human diseases (Cargill et al. 1999; Chakravarti 1999). A total of ten cSNPs were identified in each of the *MUC3A* and *MUC3B* genes, a frequency of one cSNP per 261 and 255bp, respectively (Table 4). In the Southern blotting analysis we reported recently (Kyo et al. 1999), we identified three common alleles of *MUC3A* in the Japanese population (A7, A12 and

A14) and four in a Caucasian population (a3, a5, a6 and a9). cSNPs 771T, 1005C, 2021C, 2205G, 2557A/C were all observed almost exclusively in the A7 allele in Japanese samples (98.6% of 510 common alleles examined) and seemed to be almost specific to A3 in Caucasian samples (99.0% of 100 common alleles examined). Likewise, 465C and 1065T were linked to the A12 allele, and 256C and 1398A were linked to A14. The allele frequency at each SNP site observed in UC or CD overall had no significant difference with that of control. However, subgroup analysis revealed significant associations of SNPs with familial CD: compared with control, familial CD cases (younger brothers or sisters of the CD-affected siblings) showed significantly increased frequency of carrying the A7-linked alleles, which were reproduced in the group of elder brothers or sisters of the CD-affected siblings (Table 5). We examined ages at onset in CD-affected siblings to investigate whether the observed associations have practical implications for the risk of CD. Figure 4 shows the distribution of their ages at onset, with various genotypes at nucleotide 2557 in *MUC3A*, demonstrating a clear tendency of earlier onset in those who have nucleotides A or C at this position. The shift of onset to younger ages seems to be gene-dose dependent, and the mean age at onset is significantly younger in patients with the genotype XX (X denotes nucleotide A or C)

Table 4. Frequencies of SNPs and nucleotide diversities of the 3' regions of *MUC3A* and *MUC3B*

Region	bp Screened	No. SNPs ^a	Frequency (/bp) ^a	Nucleotide diversity ($\pi \times 10^4$)
<i>MUC3A</i>				
Coding	2609	10 (19)	1/261 (1/137)	15.3
Noncoding	1088	1 (5)	1/1088 (1/218)	1.2
Overall	3697	11 (24)	1/336 (1/154)	11.2
<i>MUC3B</i>				
Coding	2548	10 (10)	1/255 (1/255)	11.1
Noncoding	1218	8 (8)	1/152 (1/152)	24.8
Overall	3766	18 (18)	1/209 (1/209)	15.5

^aNumbers in parentheses indicate the numbers or frequencies of sequence variations which include rare variants

Table 5. Genotypes at nt 2557 of *MUC3A*

Genotype	Control (%)	UC (%)	CD ^a (%)	CD-sporadic	CD-sib (1) ^b	CD-sib (2) ^c
TT	42 (31.8)	21 (29.2)	16 (22.1)	12 (27.3)	4 (15.4)	4 (15.4)
AT + CT	66 + 1 (50.8)	34 + 3 (51.4)	33 + 3 (51.9)	23 + 1 (54.5)	10 + 2 (46.2)	12 + 1 (50.0)
AA + AC	22 + 1 (17.4)	14 + 0 (19.4)	17 + 1 (26.0)	8 + 0 (18.2)	9 + 1 (38.5)	7 + 2 (34.6)
Totals	132	72	70	44	26	26
f(A,C) ^d	0.43	0.45	0.51	0.45	0.62*	0.60**

P* = 0.0132; *P* = 0.0262. Odds ratios for CD in familial occurrence are 4.34 (XX vs TT; X denotes nucleotide A or C; 95% confidence interval [CI], 1.70–11.10; *P* = 0.0022) and 1.96 (TX vs TT; 95% CI, 0.82–4.70; *P* = 0.1321)

^aCD includes CD-sporadic and CD-sib (1)

^bCD-sib (1) indicates younger brothers or sisters of the CD-affected siblings

^cCD-sib (2) indicates elder brothers or sisters of the CD-affected siblings

^df(A, C), combined frequency of A and C alleles

Fig. 4. Age at onset in Crohns disease-affected siblings (both elder and younger brothers or sisters) with various genotypes at nucleotide 2557 of *MUC3A*. X denotes nucleotide A or C, white bars, younger than 11 years; hatched bars, from 11–20 years; dotted bars, from 21–30 years; dark bars, older than 30 years, ^a*P* = 0.0670

than in patients with the genotype TT ($P = 0.0670$). The similar nucleotide variation observed at nucleotide 2557 of *MUC3A* was also observed at the corresponding position in *MUC3B* (nucleotide 2560) although nucleotides C and A were rare in *MUC3A* and *MUC3B*, respectively (Table 2). However, the association observed at nucleotide 2557 in *MUCA* was not reproduced at nucleotide 2560 in *MUC3B* (combined frequency of A and C alleles: 55.8% in younger brothers or sisters of CD-affected siblings vs 55.3% in control; $P = 0.9507$), suggesting that a common variant of *MUC3A* and not of *MUC3B* may confer genetic predisposition to CD.

Discussion

Twelve human mucin genes have so far been identified, but only five of them have been fully sequenced at the cDNA level. Mucin genes contain in their coding regions long stretches of variable number of tandem repeats (VNTRs) covering several kilobases, and the cloning of such genes is extraordinarily difficult, primarily because such clones show great disadvantages in propagation in bacterial cells. We could not obtain any clones of “*MUC3*” in the screening of the libraries of bacterial artificial chromosomes, P1-derived artificial chromosomes, or cosmids. Such clones are sometimes tolerated in yeast cells, but no clones were obtained in the screening of the yeast artificial chromosome library. We constructed a subgenomic library by cutting out *Pvu*II-digested genomic DNA fragments of 10 to 15 kb in size and cloning them into λ dashII vector to investigate the differences in sequences or structures between common and rare alleles of “*MUC3*” (now *MUC3A*), which we reported recently (Kyo et al. 1999); however, we were unable to obtain any unrecombined clones in the screening of 8×10^6 clones, which we estimated to be equal to approximately 300 genomes. The entire structure of this “gene” remains unknown, and it has also been unknown whether the 51-bp repetitive regions lie in a single gene, making an internal duplication, or whether they lie in two tandemly arrayed genes (Gum et al. 1997). We first estimated the size of “*MUC3*” mRNA to be approximately 17 kb, based on the molecular mass of the “*MUC3*” protein precursor of 550 kDa (van Klinken et al. 1995). This was recently confirmed by Northern blot analysis, using mRNA extracted by an improved method for large mRNA, with the size of “*MUC3*” mRNA estimated to be 17.5 kb in the gastric antrum and 17 kb in the colon (Debailleul et al. 1998). However, the total size of the known repetitive and unique regions far exceeds the estimated size, which led us to hypothesize that “*MUC3*” is composed of two genes.

We first analyzed the sequences of the 3' regions just downstream of the two 51-bp repeats, and the differences observed in exonic sequences enabled us to analyze the 3' terminal structures of the two genes by conducting successive RACE reactions, using primers specific to each gene. *MUC3A* and *MUC3B* share high homology in their 51-bp repetitive regions and in the 3' regions. The recently re-

ported mucin genes *MUC11* and *MUC12* also have a very high degree of sequence similarity at their tandem repeat regions, and both are located on chromosome 7q22, the location of *MUC3A* and *MUC3B* (Williams et al. 1999b). Although the sequence of the 3' region of *MUC11* is unknown, that of *MUC12* contains two EGF-like domains and a putative transmembrane region, like *MUC3A* and *MUC3B*. The structural similarities among these clustering genes suggest that their 3' regions may be derived from a common ancestral gene by a DNA shuffling mechanism, as suggested in secretory mucin genes clustering on 11p15 (Buisine et al. 1998), and that these genes on 7q22 may share some common functional features. The functional importance of these domains in *MUC3A* and *MUC3B* is supported by the presence of EGF1 in all transcripts of *MUC3A* and *MUC3B* and by the presence of EGF2 exclusively in all of the membrane-bound forms, and this presence of EGF2 suggests that it may function by interacting with other membrane proteins. The EGF-like motifs have the same cysteine distribution that is well conserved among functional EGF-bearing proteins involved in the adhesion, growth, differentiation, and migration of various epithelial cells (Appella et al. 1988; Engel 1989). These proteins are growth factors, receptors or receptor-like proteins, suggesting that the EGF-like regions are responsible for the interactions with a receptor or a ligand (Appella et al. 1988). The structures and the alignments of these EGF motifs observed in *MUC3A* and *MUC3B* are very close to those of *MUC4* and rat *ASGP2* (a rat homologue of human *MUC4*; Moniaux et al. 1999), both of which also encode membrane-bound mucins, and EGF1 of rat *ASGP2* has been shown to act as a ligand for the receptor tyrosine kinase p185^{neu} (Carraway et al. 1999). p185^{neu}, which shows similarity with the EGF receptor, is a rat homologue of the proto-oncogene *c-erbB2*, and is a potential modulator of cell growth. Several EGF-like peptides have so far been identified in the gut, and the structural similarities of the 3' regions of *MUC3A* and *MUC3B* with those of *MUC4* and *ASGP2* suggest that *MUC3A* and *MUC3B* proteins may also function as peptide growth factors. Furthermore, intestinal mucin glycoproteins are shown to promote epithelial restitution, that is, cell migration to the injured area, in the initial response to mucosal injury, both alone and in combination with trefoil factors in a synergistic manner (Kindon et al. 1995), which may be attributable to the function of those genes clustering on 7q22. Thus, *MUC3A* and *MUC3B* may be multifunctional cell-surface glycoproteins, like *MUC1* and *MUC4* (Agrawal et al. 1998a; Moniaux et al. 1999). The high level of expression of *MUC3A* even in those tissues which have no known mucin secretion may indicate the importance of this protein as a cytokine.

We reported that individuals carrying one or two rare alleles of *MUC3* (now *MUC3A*), which have an unusual number of 51-bp repeat units, have an increased risk of UC, with an odds ratio of 2.64 (95% confidence interval [CI], 1.60–4.33; $P = 0.0001$; Kyo et al. 1999). In control populations, common alleles accounted for more than 95% of total alleles, and almost all of the rare alleles accounted for less than 1%, and should be considered as mutations rather than

polymorphisms (Richards and Sutherland 1992). Some of the 51-bp repeat units are known to contain 18-bp interrupting sequences (Gum et al. 1990), which may function to stabilize the configuration of the repeat domain (Richards and Sutherland 1992) as suggested by the presence of interruptions of CAT within the (CAG)_n repeat of the gene for spinocerebellar ataxia type 1 (Chung et al. 1993) and of CCT within the (CCG)_n repeat of the gene for fragile X syndrome (Verkerk et al. 1991; Richards and Sutherland 1992). The presence of interrupting sequences and the fact that more than 95% of alleles belonged to common alleles suggest the importance of structural stability of the 51-bp repeat domain, and it seems plausible that these rare alleles could encode mucin proteins with conformational alterations. Because O-glycosylation as well as N-glycosylation is dependent on the structure of the mucin protein (van den Steen et al. 1998), the conformational alteration of the protein could cause underglycosylation, which results in increased sensitivity for proteases, especially those secreted by intestinal bacteria (van den Steen et al. 1998; Variyam and Hoskins 1981). The degradation of the mucin protein could disrupt its epithelial barrier function, a known physical role of mucin, as well disrupting the epithelial restitution and proliferation effects mentioned above. *Muc1* is one of the membrane-bound mucins expressed in the murine conjunctiva, and *Muc1* null mice are markedly predisposed to develop bacterial conjunctivitis, suggesting the critical protective role of mucin especially against bacteria (Kardon et al. 1999). As “*MUC3*” was considered to be a secretory mucin (Gum et al. 1997) when we reported the association with UC, we could not explain the dominant effect of rare alleles, because mucin protein encoded by a common allele would compensate for the function of degraded mucin; for example, by transcriptional regulation. Now that *MUC3A* is shown to be a membrane-bound mucin, the increased risk of UC in those who have one or two rare alleles of *MUC3A* is easy to understand because of the limited expression of the protein within the cell membrane. Normal colonic mucin is heavily sulfated, a feature that prevents degradation by bacterial proteases, and the significant loss of mucin sulfation observed in patients with UC (Raouf et al. 1992) may also be related to the underglycosylation of *MUC3A* protein.

SNPs, particularly those that alter amino acid sequences, can be the etiologic cause of the person-to-person differences in disease susceptibility. To identify variants involved in the susceptibility to IBDs, we surveyed, for the identification of SNPs, the exons of the 3' regions of *MUC3A* and *MUC3B*. The overall estimates of nucleotide diversities (π) were two to four times higher in *MUC3A* and three to five times higher in *MUC3B* than those observed in other studies (Chakravarti 1999; Cargill et al. 1999). A number of factors may contribute to the high level of diversities: (i) the presence of long stretches of repeated sequences (Chakravarti 1998), (ii) local recombination frequencies (Begun and Aquadro 1992; Nachman et al. 1998), and (iii) gene conversion events between *MUC3A* and *MUC3B*, as suggested by the similar sequence variations observed in the two genes (Table 2). Using these SNPs in the association

study, we found that A7-linked variants of *MUC3A* may confer genetic predisposition to CD. Among the polymorphisms which were associated with CD, 2557T (853Y) gives a YVAL motif in the cytoplasmic tail (at residue 853–856 of *MUC3A*; Fig. 2d) which is similar to specific motifs recognized by SH-2 domain-containing proteins (Songyang et al. 1994). *MUC12* also contains a similar motif (YNNF sequence in the cytoplasmic tail (Williams et al. 1999b), suggesting that cell signaling via SH-2-domain-containing proteins may be a common feature of the mucin proteins encoded by those genes clustering on 7q22. Thus, the *MUC3A* allele with this tyrosine residue may show a protective effect against CD by this signal transduction. The association was observed only in familial CD, but not in CD overall, suggesting that both genetic and environmental factors are involved in the risk for the disease occurrence, and that there is a strong environmental effect particularly in the occurrence of sporadic CD. CD-affected siblings could share some of the major and minor susceptibility genes, and the A7-linked allele of *MUC3A* may act in combination with the major determinant gene for disease occurrence and its earlier onset. We cannot rule out completely the possibility that the association may result from linkage disequilibrium with another intragenic polymorphism on the A7 allele or another gene near *MUC3A* that confers susceptibility to CD. The latter possibility, however, is not plausible considering the clear tendency of the shift of onset to younger ages and the high frequencies of the minor alleles. As the relative frequency of each allele reflects, on average, its time of origin, the high frequencies of minor alleles indicate the long duration (more than 10,000 years; Chakravarti, 1999) since the occurrence of mutations at these sites. This is supported by the presence of these variations in Caucasian samples, suggesting that these mutations occurred before the differentiation of the Asian and the Caucasian populations. The extent of linkage disequilibrium at this locus is expected to be small because of the expected long duration, because the occurrence of these mutations and the high value of the nucleotide diversity (π) suggest frequent occurrence of meiotic recombination at this locus. Thus, it is not likely for another gene near *MUC3A* to show linkage disequilibrium with such a clear tendency to shift the onset to younger ages. We identified IBD patients with one or more mutations in the 3' region of *MUC3A*, and the frequency of individuals with mutations was significantly higher in IBD patients than in controls. As for the mutations in the coding region, one patient with CD had seven mutations in CEX. I of *MUC3A*, and five of them altered the amino acid sequences. Other mutations in the coding region also altered the amino acid sequences and resulted in charge differences. These mutations or rare variants are typically recent in origin, with population-specificity, lower survival, and stronger selection pressure, and are likely to have a direct impact on the protein function (Cargill et al. 1999; Chakravarti 1999; Halushka et al. 1999), which could bear on susceptibility to IBDs, particularly to CD. Thus, both common and rare variants that are likely to influence the function of *MUC3A* mucin protein may be involved in the susceptibility to CD.

In this study, we demonstrated that distinct variants of *MUC3A* may be involved in the occurrence of UC and CD. cSNPs and mutations that alter amino acid sequences of the 3' region of *MUC3A* could probably influence the function of this mucin protein, which would, in turn, cause increased susceptibility to CD. Rare variants of *MUC3A*, which have an unusual number of 51-bp repeat units, could have a defect in the barrier function of this mucin protein and would confer genetic predisposition to UC. Various genetic and environmental factors could be related to the risk of IBDs, and discovering a susceptibility gene in such a multifactorial disorder will not only elucidate the molecular basis of the pathogenesis but will aid in the investigation of environmental factors. The identification of common environmental factors in those who have a common genetic factor will, in turn, help genetically susceptible people to prevent development of the disease by avoiding the environmental triggers, and may also offer a new strategy for the treatment of IBDs.

References

- Agrawal B, Gendler SJ, Longenecker BM (1998a) The biological role of mucins in cellular interactions and immune regulation: prospects for cancer immunotherapy. *Mol Med Today* 4:397–403
- Agrawal B, Krantz MJ, Reddish MA, Longenecker BM (1998b) Cancer-associated MUC1 mucin inhibits human T-cell proliferation, which is reversible by IL-2. *Nat Med* 4:43–49
- Appella EA, Weber IT, Blasi F (1998) Structure and function of epidermal growth factor-like regions in proteins. *FEBS Lett* 231:1–4
- Begun DJ, Aquadro CF (1992) Levels of naturally occurring DNA polymorphism correlate with recombination rates in *D. melanogaster*. *Nature* 356:519–520
- Buisine MP, Desseyn JL, Porchet N, Degand P, Laine A, Aubert JP (1998) Genomic organization of the 3'-region of the human *MUC5AC* mucin gene: additional evidence for a common ancestral gene for the 11p15.5 mucin gene family. *Biochem J* 332:729–738
- Buisine MP, Desreumaux P, Debailleul V, Gambiez L, Geboes K, Ectors N, Delescaut MP, Degand P, Aubert JP, Colombel JF, Porchet N (1999) Abnormality in mucin gene expression in Crohn's disease. *Inflamm Bowel Dis* 5:24–32
- Cargill M, Altshuler D, Ireland J, Sklar P, Ardlie K, Patil N, Lane CR, Lim EP, Kalyanaraman N, Nemesh J, Ziaugra L, Friedland L, Rolfe A, Warrington J, Lipshutz R, Daley GQ, Lander ES (1999) Characterization of single-nucleotide polymorphisms in coding regions of human genes. *Nat Genet* 22:231–238
- Carraway KL III, Rossi EA, Komatsu M, Price-Schiavi SA, Huang D, Guy PM, Carvajal ME, Fregien N, Carraway CA, Carraway KL (1999) An intramembrane modulator of the ErbB2 receptor tyrosine kinase that potentiates neuregulin signaling. *J Biol Chem* 274:5263–5266
- Chakravarti (1998) It's raining SNPs, hallelujah? *Nat Genet* 19:216–217
- Chakravarti A (1999) Population genetics – making sense out of sequence. *Nat Genet* 21:56–60
- Chung MY, Ranum LPW, Duvick LA, Servadio A, Zoghbi HY, Orr HT (1993) Evidence for a mechanism predisposing to intergenerational CAG repeat instability in spinocerebellar ataxia type 1. *Nat Genet* 5:254–258
- Crawley SC, Gum JR Jr, Hicks JW, Pratt WS, Aubert JP, Swallow DM, Kim YS (1999) Genomic organization and structure of the 3' region of human *MUC3*: alternative splicing predicts membrane-bound and soluble forms of the mucin. *Biochem Biophys Res Commun* 263:728–736
- Debailleul V, Laine A, Huet G, Mathon P, d'Hooghe MC, Aubert JP, Porchet N (1998) Human mucin genes MUC2, MUC3, MUC4, MUC5AC, MUC5B, and MUC6 express stable and extremely large mRNAs and exhibit a variable length polymorphism; an improved method to analyze large mRNAs. *J Biol Chem* 273:881–890
- Engel J (1989) EGF-like domains in extracellular matrix proteins: localized signals for growth and differentiation? *FEBS Lett* 251:1–7
- Gum JR, Hicks JW, Swallow DM, Lagace RL, Byrd JC, Lamport DTA, Siddiki B, Kim YS (1990) Molecular cloning of a novel human intestinal mucin gene. *Biochem Biophys Res Commun* 171:407–415
- Gum JR Jr, Ho JLL, Pratt WS, Hicks JW, Hill AS, Vinall LE, Robertson AM, Swallow DM, Kim YS (1997) MUC3 human intestinal mucin: analysis of gene structure, the carboxyl terminus, and a novel upstream repetitive region. *J Biol Chem* 272:26,678–26,686
- Halushka M, Fan JB, Bentley K, Hsie L, Shen N, Weder A, Cooper R, Lipshutz R, Chakravarti A (1999) Patterns of single-nucleotide polymorphisms in candidate genes for blood-pressure homeostasis. *Nat Genet* 22:239–247
- Housman D, Ledley FD (1998) Why pharmacogenomics? Why now? *Nat Biotechnol* 16:492–493
- Kardon R, Price RE, Julian J, Lagow E, Tseng SC, Gendler SJ, Carson DD (1999) Bacterial conjunctivitis in Muc1 null mice. *Invest Ophthalmol Vis Sci* 40:1328–1335
- Kindon H, Pothoulakis C, Thim L, Lynch-Devaney K, Podolsky DK (1995) Trefoil peptide protection of intestinal epithelial barrier function: cooperative interaction with mucin glycoprotein. *Gastroenterology* 109:516–523
- Kyo K, Parkes M, Takei Y, Nishimori H, Vyas P, Satsangi J, Simmons J, Nagawa H, Baba S, Jewell D, Muto T, Lathrop GM, Nakamura Y (1999) Association of ulcerative colitis with rare alleles of the human intestinal mucin gene, *MUC3*. *Hum Mol Genet* 8:307–311
- Ligtenberg MJ, Kruijsaar L, Buijs F, van Meijer M, Litvinov SV, Hikens J (1992) Cell-associated episialin is a complex containing two proteins derived from a common precursor. *J Biol Chem* 267:6171–6177
- Moniaux N, Nollet S, Porchet N, Degand P, Laine A, Aubert JP (1999) Complete sequence of the human mucin MUC4: a putative cell membrane-associated mucin. *Biochem J* 338:325–333
- Nachman MW, Bauer VL, Crowell SL, Aquadro CF (1998) DNA variability and recombination rates at X-linked loci in humans. *Genetics* 150:1133–1141
- Nickerson DA, Tobe VO, Taylor SL (1997) PolyPhred: automating the detection and genotyping of single nucleotide substitutions using fluorescence-based resequencing. *Nucleic Acids Res* 25:2745–2751
- Orholm M, Munkholm P, Langholz E, Nielsen OH, Sorensen TIA, Binder V (1991) Familial occurrence of inflammatory bowel disease. *N Engl J Med* 324:84–88
- Raouf AH, Tsai HH, Parker N, Hoffman J, Walker RJ, Rhodes JM (1992) Sulphation of colonic and rectal mucin in inflammatory bowel disease: reduced sulphation of rectal mucus in ulcerative colitis. *Clin Sci* 83:623–626
- Richards RI, Sutherland GR (1992) Dynamic mutations: a new class of mutations causing human disease. *Cell* 70:709–712
- Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
- Satsangi J, Parkes M, Louis E, Hasimoto L, Kato N, Welsh K, Terwilliger JD, Lathrop GM, Bell JI, Jewell DP (1996) Two-stage genome-wide search in inflammatory bowel disease provides evidence for susceptibility loci on chromosomes 3, 7, 12. *Nat Genet* 14:199–202
- Songyang Z, Shoelson SE, McGlade J, Olivier P, Pawson T, Bustello XR, Barbacid M, Sabe H, Hanafusa H, Yi T, Ren R, Baltimore D, Ratnoffsky S, Feldman RA, Cantley LC (1994) Specific motifs recognized by the SH2 domains of Csk, 3BP2, fps/fes, GRB-2, HCP, SHC, Syk, and Vav. *Mol Cell Biol* 14:2777–2785
- Tysk C, Lindberg E, Jarnerot G, Floderus-Myrhed B (1988) Ulcerative colitis and Crohn's disease in an unselected population of monozygotic and dizygotic twins. A study of heritability and the influence of smoking. *Gut* 29:990–996
- Tysk C, Riedesel H, Lindberg E, Panzini B, Podlasky D, Jarnerot G (1991) Colonic glycoproteins in monozygotic twins with inflammatory bowel disease. *Gastroenterology* 100:419–423
- van den Steen P, Rudd PM, Dwek RA (1998) Concepts and principles of O-linked glycosylation. *Crit Rev Biochem Mol Biol* 33:151–208
- van Klinken BJ, Oussoren E, Weenink JJ, Strous GJ, Buller HA, Dekker J, Einerhand AW (1995) The human intestinal cell lines

- Caco-2 and LS174T as models to study cell-type specific mucin expression. *Glycoconj J* 13:757–768
- van Klinken BJW, van Dikken TC, Oussoren E, Buller HA, Dekker J, Einerhand AWC (1997) Molecular cloning of human MUC3 cDNA reveals a novel 59 amino acid tandem repeat region. *Biochem Biophys Res Commun* 238:143–148
- Variyam EP, Hoskins LC (1981) Mucin degradation in human colon ecosystems. Degradation of hog gastric mucin by fecal extracts and fecal cultures. *Gastroenterology* 81:751–758
- Verkerk AJ, Pieretti M, Sutcliffe JS, Fu YH, Kuhl DP, Pizzuti A, Reiner O, Richards S, Victoria MF, Zhang F, Eussen BE, van Ommen GJB, Blonden LAJ, Riggins GJ, Chastain JL, Kunst CB, Galjaard H, Caskey CT, Nelson DL, Oostra BA, Warren ST (1991) Identification of a gene (*FMR-1*) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in Fragile X syndrome. *Cell* 65:95–914
- Williams SJ, Munster DJ, Quin RJ, Gotley DC, McGuckin MA (1999a) The *MUC3* gene encodes a transmembrane mucin and is alternatively spliced. *Biochem Biophys Res Commun* 261:83–89
- Williams SJ, McGuckin MA, Gotley DC, Eyre HJ, Sutherland GR, Antalis TM (1999b) Two novel mucin genes down-regulated in colorectal cancer identified by differential display. *Cancer Res* 59:4083–4089