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Identification of novel genes that are differentially expressed in human colorectal carcinoma

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Abstract

By using mRNA differential display technology, we have identified three cDNA clones, myl 3, myl 4, and myl 6, to show significant changes in expression between human colorectal tumor and paired normal tissue. Northern blot analysis indicated that clone myl 3 expression was elevated in normal tissue, and clone myl 4 expression was elevated in tumor tissue. Nucleotide sequence analysis revealed that clones myl 3 and myl 4 have not been previously identified. However, clone myl 6 appears to be the human homolog of the 3′ end region of tissue inhibitor of metalloproteinase 3 (TIMP-3). Northern blot analysis showed that a 2.5 kb TIMP-3 transcript was expressed at a much higher level in normal tissue than the colorectal tumor.

Keywords: Colorectal tumor; Reverse transcriptase–polymerase chain reaction; mRNA differential display; Tissue inhibitor of metalloproteinase 3

Searching for colorectal cancer-specific biomarkers has been an important issue, since such markers will shed light on early detection, prognosis, and treatment of the disease. The tests for occult blood in the feces are widely used to screen for colorectal cancer. However, better means of diagnosis and prognosis for patients with colorectal cancer are needed to improve the survival rate, and efficiency of postoperative adjuvant therapy [1,2]. Colorectal cancer results from the accumulation of several distinct genetic alterations involving chromosomal translocations, gene amplifications and point mutations in oncogenes and suppressor genes [3–6]. In a recent study, an increasing expression of the *ras* oncogene and partial deletion of p53 gene in colorectal tumor tissue has been reported [7–9]. Also, a comprehensive study of human cancer done by Goldstein et al. showed that colon cancer had relatively high multiple drug-resistant gene (*mdr*) expression [10]. However, early detection of colorectal cancer by monitoring *ras*, p53, and *mdr* expression levels are still unsatisfactory due to either their low sensitivity or low specificity.

In this study, we used mRNA differential display reverse transcription–polymerase chain reaction (DDRT–PCR) [11,12] to search for the genes that are differentially expressed in colorectal tumor and paired normal tissues, and evaluated whether they are useful as potential biomarkers for colorectal cancer.

The expression of mRNA in colorectal tumor classified as a Dukes' D stage and paired normal tissue were analyzed using combinations of 40 random 10-mers and three $T_{11}N$ primers. Each primer combina-

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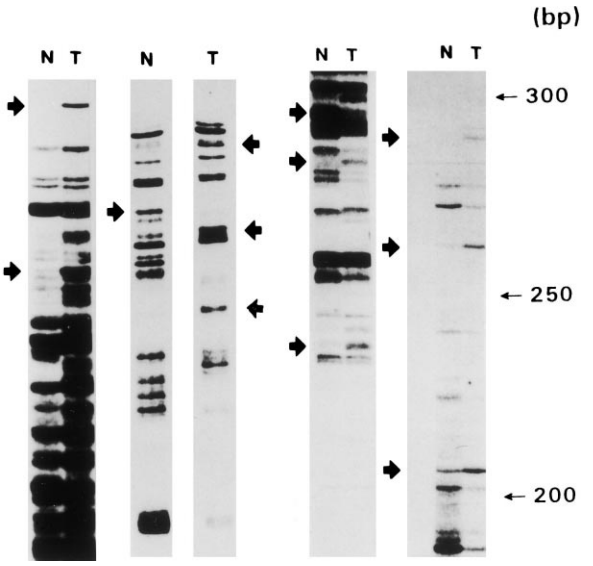

Fig. 1. Autoradiogram of differential mRNA display of candidate clones. Total DNA-free RNA was isolated from tissue by TRIzol reagent-chloroform (Gibco) extraction [13], and was reverse transcribed in 1×MMLV buffer which contained 2 μM of either $T_{11}A$, $T_{11}G$, or $T_{11}C$ as the 3′-primer oligonucleotide and Superscript-II (Gibco). After RT reaction, PCR was performed by adding dNTPs, 5 μC_i [^{35}S]dATP, $T_{11}N$ oligonucleotide, multiple arbitrary 10-mer oligonucleotide sets (Genosys Biotechnologies), and *Taq* DNA polymerase (Promega). PCR reactions were performed in the Rapidcycler (Idaho Technology) at 94°C for 30 s and 40 cycles at 94°C for 2 s, 40°C for 40 s, 72°C for 30 s, followed by 72°C for 5 min. PCR products were electrophoresed on 6% acrylamide DNA sequencing gels under denaturing conditions. Twelve candidate cDNA fragments which show altered expression by differential mRNA display are marked by arrowheads and were selected for subcloning. Lane N indicates normal tissue; lane T indicates tumor tissue.

tion displayed 80–150 bands, allowing approximately 10 000 different mRNA species to be screened in this study. As shown in Fig. 1, 12 candidate clones exhibited differences in their expression between normal and colorectal tumor tissues. These changes were confirmed by repeating the PCR and differential display procedures.

The isolated clones from the display gel were reamplified by PCR, and were subcloned into pGEM-T vector, and the clones were used as probes for Northern blot analysis. By Northern blot analysis using total RNA extracted from tissue samples, three clones (myl 3, myl 4, and myl 6) were found to hybridize with genes which were differentially expressed between normal and colorectal tumor tissues. As shown in Fig. 2, two transcripts (1.7 kb and 3.2 kb) probed by clone myl 3 were present in normal tissue but not in tumor tissue. Two transcripts (1.8 kb and 3.4 kb) probed by clone myl 4 were present in tumor tissue but not in normal tissue. When clone myl 6 was used as a probe, two predominant transcripts (4.5 kb and 5.5 kb) were found to have the same levels of expression in both normal and tumor tissues. However, two other transcripts (1.7 kb and 2.5 kb) were found to have much higher levels in normal tissue than in tumor tissue. Several possibilities can explain the occurrence of multiple transcripts, including alternative utilization of polyadenylation sites, alternative splicing of the message, or the presence of extended 5′ untranslated regions. The availability of full length of those cDNA clones and the future availability of large amounts of colorectal tumor samples will allow studies to determine if these novel genes or their coding proteins can be the biomarkers for colorectal cancer.

The nucleotide sequences of clones myl 3, myl 4, and myl 6 are shown in Fig. 3. Both myl 3 and myl 6 clones have variant polyadenylation signals. A search of GenBank/EMBL databases revealed that clone myl 6 had 98% homology to the tissue inhibitor of metalloproteinase 3 (TIMP-3) [14,15]. The sequence of cDNA fragment is limited because of primer de-

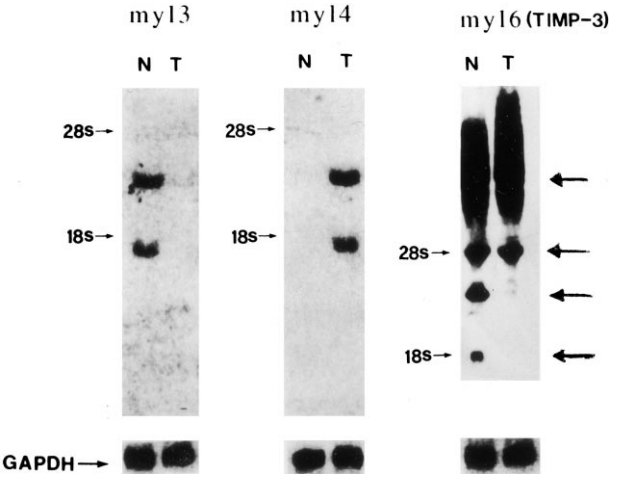

Fig. 2. Northern blot analysis using [^{32}P]dATP-labeled cDNA probe derived from PCR reamplification of clones myl 3, myl 4, and myl 6. The same blots reprobed with human glyceraldehyde-3-phosphate (GAPDH) cDNA as a loading control. Each lane represents 10 μg of total cytoplasmic RNA isolated from normal or tumor tissue. Arrows indicate the position of transcripts present in colorectal tissues.

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clone myl 3      Length: 262 bp
     AATTTTTTTT AATCGTGTGT TTAATTTTAC ATCAACCATA TAGCATATAT   50
     TTAGCAATAT GACAGTCTAC TGATTTTGGC TTTGTTAGAA TAGAGAATAA  100
     GTGCAAATAA TTAAATCCTA TGGCTGTATT AACAAGGGGG CCATTGATTA  150
     TAAAACTTGA ACTCAGGTCC TTTTATAAAC ATTGTTGGTA TTTAACTTTA  200
     CTTCCCATTA CTGTGGATCT AACCAAATAA TTATGTGTTT TGTGTCGCGA  250
     AAAAAAAAAA AA                                          262

clone myl 4      Length: 283 bp
     CAGCATACAG ATCTTTCACT TCCTTGGTTG GATTTATTTG CAGATATTTT   50
     TTGTAGCTAT TGTAAATGAC ATTGTTTTCT TGATTTCTTT GTTAGGGTAT  100
     AGAAATGCTA CAGATTGTTG TATGTTAATT TTATATCCTG CAACTTTACT  150
     GATTTCATTT ATTCTAACAT CTTATTGGTG GAATCTTTAG GGTTTTCTAT  200
     AAGATCATGT CATCTGTAAA CAGGGACAAT TTAAGTTCTT CCTTTCCAAT  250
     TTGGATACCT TTTATTTCGA AAAAAAAAAA AAA                    283


clone myl 6      Length: 256 bp
     CCCTATAAAA AGTGAACATT ATGCAACATT ACAAGACAAT ATACATTCAC   50
     GGAATATAAA ATTCATAAAT AACATGGTGG AAAACTGTAA ACAGTGCTAC  100
     GAAATTTAGC AACAAATACA TTCCTTCTAG ACAGGGTTCG AGATCTCTTG  150
     TTGGTTTCTC TCCATCACTT CTGGGTTTCA GGACAGCAGA CTGGCTAAAG  200
     GGAAAGGCGG ATGCTGGGAG AATCTAAGAA GCCTCTACCC CCAAAAAAAA  250
     AAAAAA                                                 256
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Fig. 3. Nucleotide sequence of clones myl 3, myl 4, and myl 6. DNA sequencing was performed directly from the TA cloning vector using Sequenase version 2.0 kit (US Biochemical). T7 and SP6 promoter primers were used to locate the cloned insert. DNA sequences were analyzed by the FASTA search program of GCG package and were screened for homology against the GenBank/EMBL database sequences. All contain a poly A tail and a variant polyadenylation signal (underlined) except clone myl 4.

signs inherent to the DDRT–PCR method that result in production of short cDNA sequences hybridized with the 3′ end region of target genes. Therefore, the lack of homology for myl 3 and myl 4 clones with known sequences does not eliminate the possibility that they may be homologous to known genes or gene families, since non-coding 3′ regions of mRNA species show great species to species variation. Further work to clone the full-length cDNAs of these genes is needed to clarify the identity of nucleotide sequences and to speculate regarding their functions in colorectal tumorigenesis.

To confirm the differential expression of TIMP-3 between human colorectal tumor and normal tissues, a cDNA specific for TIMP-3 open reading frame fragment (390–791 bp) generated by RT–PCR from total RNA of colon tissue was used as a probe in the Northern blot analysis. It was observed that transcript 2.5 kb was predominantly present in the normal tissues but poorly expressed in tumor tissues (Fig. 4). We speculated that the different transcript pattern observed from the Northern blot analysis by using a downstream DNA region (myl 6) and a DNA open reading frame region as probes was due to alternative splicing of the TIMP-3 gene.

TIMP-3 has been reported to inhibit collagenases and gelatinases [14,15]. Metalloproteinases are able to degrade the different components of extracellular matrix, thereby facilitating tumor growth, invasion, and metastasis [16,17]. Therefore, overproduction of TIMP-1 and TIMP-2, members of the TIMP family, could suppress tumor metastasis in vitro and in vivo [18,19]. Conversely, down-regulation of TIMP-1 by antisense RNA converts non-tumorigenic cells into malignant cells [20]. Although the analysis of TIMP-3 differential expression is more confirmatory than novel expression [21–23], in the present study

Table 1
Incidence of gene expression in ten colorectal tumors and normal colorectal tissues by using clone myl 3, myl 4, and TIMP-3 cDNA fragment (401 bp) as probes

	myl 3	myl 4	TIMP-3
Tumor	2/10 (20%)	7/10 (70%)	0/10
Normal	8/10 (80%)	0/10	6/10 (60%)

Colorectal tumor samples were collected at surgery. Samples were dissected into two parts; one was for routine histopathological evaluation, the other was frozen immediately in liquid nitrogen after surgical resection and maintained at −70°C until RNA extraction. The paired normal colon tissue samples were dissected at locations 10 cm away from tumors.

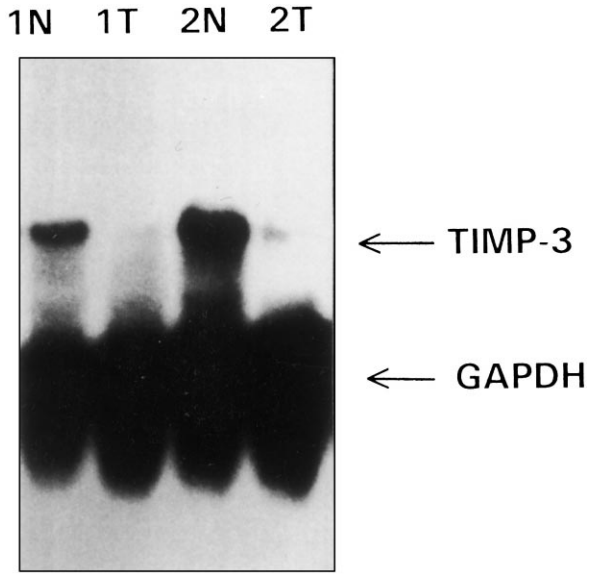

Fig. 4. Northern blot analysis using a [^{32}P]dATP-labeled cDNA probe derived from reamplification of a 401 bp TIMP-3 and a GAPDH probe. The TIMP-3 cDNA fragment with a length of 401 bp was generated by PCR of cDNA from the colon tissue by using forward (F = 5′AACTCCGACATCGTGATCCGGG-CC3′) and reverse (R = 5′GGAGAGCATGTCGGTCCAGAG-AC3′) primers which spanned region 390–791 of the published TIMP-3 sequence [14]. The PCR product of TIMP-3 was further identified by sequencing both strands with the dideoxy chain termination method using the Sequenase 2.0 system (US Biochemical). Each lane represents 10 μg of total cytoplasmic RNA isolated from normal colorectal (N) or tumor tissue (T).

the 2.5 kb TIMP-3 gene expressed at a higher level in normal tissue than in colorectal tumor is novel. Further studies are needed to clarify the mechanism of action of TIMP-3 and cellular physiological consequences of altered TIMP-3 expression in colorectal cancer metastasis.

In a study of ten colorectal carcinomas, we used clones TIMP-3 cDNA, myl 3, and myl 4 as probes for Northern blot analysis. Tumor tissues and matched normal colon tissues were obtained from ten consecutive, non-selected patients (six males and four females) whose age ranged from 45 to 66 years old. Of the ten carcinoma tissues, three were primary tumors and seven were from metastasis to the liver. A pathological diagnosis of each tumor was performed according to the criteria proposed by the World Health Organization and UICC. The result showed that the probe myl 4 detected gene expression in seven tumors (70%) but did not detect any expression in the corresponding normal colorectal tissue (Table 1). On the other hand, we detected high gene expression in eight normal colorectal tissues (80%) and only weak gene expression in two tumors by using clone myl 3 as a probe. By using TIMP-3 cDNA fragment as a probe, Northern blot analysis showed that a 2.5 kb gene was highly expressed in six normal tissues but not in the corresponding tumor tissues.

In conclusion, although the fundamental questions related to function of those genes in tumorigenesis are not answered, TIMP-3, myl 3, and myl 4 hybridized genes can begin to be assessed for possible use in clinical diagnosis. At the current stage, we are conducting a large pilot study by using these novel genes as probes to evaluate their potential to be used as biomarkers for colorectal cancer.

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