

Alterations of Gene Expression during Colorectal Carcinogenesis Revealed by cDNA Microarrays after Laser-Capture Microdissection of Tumor Tissues and Normal Epithelia¹

Osamu Kitahara, Yoichi Furukawa, Toshihiro Tanaka,² Chikashi Kihara, Kenji Ono, Renpei Yanagawa, Marcelo E. Nita, Toshihisa Takagi, Yusuke Nakamura, and Tatsuhiko Tsunoda^{2, 3}

Human Genome Center, Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan

Abstract

To identify a set of genes involved in the development of colorectal carcinogenesis, we compared expression profiles of colorectal cancer cells from eight tumors with corresponding noncancerous colonic epithelia using a DNA microarray consisting of 9216 human genes. These cell populations had been rendered homogeneous by laser-capture microdissection. Expression change in more than half of the tumors was observed for 235 genes, *i.e.*, 44 up-regulated and 191 down-regulated genes. The differentially expressed genes include those associated with signal transduction, metabolizing enzymes, production of reactive oxygen species, cell cycle, transcription, mitosis, and apoptosis. Subsequent examination of 10 genes (five up-regulated and five down-regulated) by semiquantitative reverse transcription-PCR using the eight tumors together with an additional 12 samples substantiated the reliability of our analysis. The extensive list of genes identified in these experiments provides a large body of potentially valuable information of colorectal carcinogenesis and represents a source of novel targets for cancer therapy.

Introduction

Colorectal carcinoma is one of the most common types of cancer in developed countries. Despite several advances in treatment, this disease remains a threat to life for a large number of people. Clinical trials of new chemotherapeutic agents are ongoing, and drug effectiveness is improving; however, a complete cure for patients with advanced colorectal cancer awaits new targets and strategies. To achieve this goal, a comprehensive understanding of the mechanisms of colorectal carcinogenesis will be essential.

Those mechanisms have been under intensive study for the last two decades. At the molecular level, activation of oncogenes (1) and inactivation of tumor suppressor genes (2–4) are processes known to be involved in colorectal carcinogenesis. Additionally, abrogation of mismatch repair systems (5, 6) contributes to some colorectal cancers. Nevertheless, exactly how those genetic alterations bring about the development and progression of colorectal carcinomas remains to be resolved. To complicate the picture, accumulations of mutant genes in neoplasms tend to be accompanied by other genetic and epigenetic changes including loss of heterozygosity, inactivation of important genes by methylation (7) or loss of imprinting (8), and/or gene amplifications, all of which can alter gene expression profiles. There-

fore, genome-wide monitoring of gene expression is of great importance if we are to disclose the numerous and diverse events associated with carcinogenesis.

Several approaches, *e.g.*, differential hybridization, subtractive hybridization, and differential display, have been developed to identify genes that are expressed differently between normal and cancerous tissues. Zhang *et al.* (9) have contributed a method called serial analysis of gene expression (SAGE) to examine the frequency of individual transcripts using sequencing tags corresponding to specific genes, a method that enables us to analyze expression profiles in various tissues. However, because all of these methods are labor intensive and time consuming and require large amounts of mRNA, it is difficult to apply them for analysis of thousands of genes in a limited amount of clinical material.

Hence, we have recruited a newly developed technique, the cDNA microarray, that provides high-throughput analysis of expression profiles by means of small-array slides spotted with cDNAs (10–16). To detect exact differences between cancerous cells and noncancerous cells, we first separated them by means of LCM (17–19).⁴ Then, after performing T7-based RNA amplification (19), we were able to compare gene expression profiles of colorectal cancer cells with those of normal colonic epithelium using our original cDNA-microarray slides containing 9216 human cDNA fragments.

In this study, we report detection of 235 genes the expression of which was changed during development or progression of colorectal cancer. Among them, 44 showed up-regulated expression and 191 showed down-regulated expression in cancer tissues compared with noncancerous tissues. These results not only disclose the complex nature of changes in genetic activity during colorectal carcinogenesis but also provide information that should prove useful for identifying novel therapeutic targets.

Materials and Methods

Microarray Design and Production. We selected 9216 independent cDNAs including 4220 genes of known function from the UniGene database of the National Center for Biotechnology Information. Our cDNA microarray was constructed essentially as described previously (20).

Tissue Samples and Laser-Capture Microdissection. Colorectal cancer tissues and their corresponding normal mucosae were obtained with informed consent from 20 patients who underwent colectomy. Eight of these samples were embedded in TissueTek OCT medium (Sakura, Tokyo, Japan) and snap frozen at -80°C . The frozen sections were then fixed in 70% ethanol for 30 s and stained with H&E, followed by three dehydration steps of 5 s each in 70%, 95%, and 99.5% ethanol and a final 5-min dehydration in xylene. Once air-dried, the stained tissues were laser-capture microdissected by a PixCell II LCM system following the manufacturer's protocols (Arcturus Engineering,

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² Present address: SNP Research Center, RIKEN, Inside Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan.

³ To whom requests for reprints should be addressed, at Human Genome Center, Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan. Phone: 81-3-5449-5768; Fax: 81-3-5449-5568; E-mail: tatsu@ims.u-tokyo.ac.jp.

⁴ The abbreviations used are: LCM, laser-capture microdissection; aRNA, amplified RNA; RT-PCR, reverse transcription-PCR; HRS, histidyl-tRNA synthetase; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

Mountain View, CA). Normal colonic epithelial cells and carcinoma cells selectively “laser captured” ($\sim 1 \times 10^4$ cells from each sample) were estimated to be >95% homogeneous as determined by microscopic visualization (Fig. 1).

RNA Extraction, T7-based RNA Amplification, and Hybridization. Total RNAs were extracted from each sample of laser-captured cells into 350 μ l of RLT lysis buffer (Qiagen, Hilden, Germany). The extracted RNAs were treated for 1.5 h at 37°C with 10 units of DNase I (Roche, Basel, Switzerland) in the presence of 1 unit of RNase inhibitor (TOYOBO, Osaka, Japan) to remove any contaminating genomic DNA. After inactivation at 70°C for 10 min, the RNAs were purified with an RNeasy Mini Kit (Qiagen) according to the manufacturer’s recommendations. All of the DNase I-treated RNAs were subjected to T7-based RNA amplification as described previously (19). Three rounds of amplification yielded 8–40 μ g of aRNA from each sample. A 2.5- μ g aliquot of aRNA from carcinoma and that from normal colonic mucosae were labeled with Cy3-dCTP or Cy5-dCTP, respectively, by a protocol described elsewhere (20). The hybridization, washing, and scanning were carried out according to the methods described previously (20).

Data Analysis. Signal intensities of Cy3 and Cy5 from the 9216 spots were quantified and analyzed by substituting backgrounds, using ArrayVision software (Imaging Research, Inc., St. Catharines, Ontario, Canada). Subsequently, each signal was normalized so that the averaged ratio (Cy3: Cy5) of the 52 housekeeping gene signals would be 1.0, and the value of root (Cy3² + Cy5²) of each signal would be kept through the normalization. The 52 housekeeping genes were referred from “housekeeping panel” in the web site provided by Brown *et al.*⁵ Because the data were unreliable for spots with intensities below 1×10^5 relative fluorescent unit for both Cy3 and Cy5 signals, genes corresponding to those spots were not investigated any further.

Selection of Genes with Different Expression. To estimate the range of Cy3: Cy5 ratio within which the expression change could be considered as fluctuation among noncancerous cells, we first compared expression levels of each gene between noncancerous cells from different parts of the same patient. The range was determined to exclude 10% of the cases from each side (significance level $\alpha = 0.10$). When we compared the gene expression profiles between carcinoma cells and noncancerous cells, we considered genes that had a Cy3: Cy5 ratio outside this range as candidates of differentially expressed genes, up-regulated genes as those with Cy3: Cy5 \geq upper limit, and down-regulated genes as those with Cy3: Cy5 \leq lower limit. Among these candidates, genes with elevated expression in more than half the patients were selected as frequently up-regulated genes, and those with down-regulated expression in more than half the patients were selected as frequently down-regulated genes. We evaluated the significance of altered expression of the selected genes by calculating the *P*s using a permutation test to analyze whether the change of expression profile is significant or not (21). Here, we carried out a matched pair permutation between carcinoma cells and noncancerous cells for each patient because we compared those two populations in our experiment. When the samples of a patient (defined as *i*) were not permuted, we defined x_i as the expression level of carcinoma cells and y_i as the expression level of noncancerous cells from patient *i*. When the samples of a patient *i* were not permuted, we defined x_i as the expression level of noncancerous cells and y_i as the expression level of carcinoma cells from patient *i*. Because the permuted state of each patient is binary (permuted or not), we examined $2^8 = 256$ permuted cases. In each case, difference of expression $d_i = x_i - y_i$ ($i = 1 \dots 8$) of each gene was calculated. The average of difference $u = \sum d_i / 8$ and SD $\sqrt{\sum (d_i - u)^2 / 8}$ were determined. We calculated the “signal-to-noise” ratio (21) defined as $Z = u / SD$ for each case (among 256) for each gene (among 9216), which resulted in a total set of $n = (256 \times 9216)$ of *Z*-values. We estimated the probability, *P*, of a given expression pattern of an up-regulated gene from the eight patients by dividing by *n* the number of cases that showed *Z*-values greater than the calculated *Z*-value of the pattern. Similarly, we estimated the *P* of a given expression pattern of a down-regulated gene by dividing by *n* the number of cases that showed *Z*-values smaller than the calculated *Z*-value.

Semiquantitative RT-PCR. Total RNA (5 μ g) was treated with DNase I (Roche) and reversely transcribed for single-stranded cDNAs using oligo(dT)_{12–18} primer with Superscript II reverse transcriptase (Life Technologies, Inc.). Each single-stranded cDNA was diluted for subsequent PCR

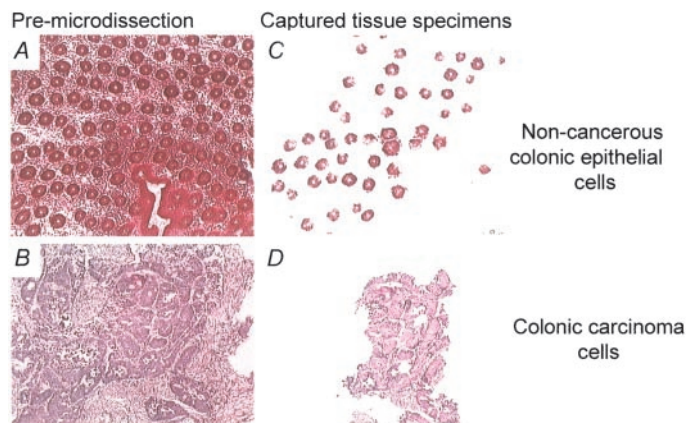


Fig. 1. Separation of cells from noncancerous and cancerous tissues by LCM. Sections (9 μ m) were stained with H&E. A, normal tissue; B, carcinoma tissue from the same patient; C, captured normal cells; D, captured carcinoma cells.

amplification by monitoring HRS as a quantitative control. HRS showed the smallest Cy3/Cy5 fluctuation among the 52 housekeeping genes in the eight experiments. Each PCR was carried out in a 50- μ l volume of $1 \times$ PCR buffer for 5 min at 95°C for initial denaturing, followed by 25–35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min, in the GeneAmp PCR system 9700 (Perkin-Elmer Applied Biosystems, Foster City, CA). The sequences of the primers used for RT-PCR were as follows: AI081175 forward, 5'-CC-CGTTTTTCCTGTATTATCTGT-3', reverse, 5'-CTCTTCTTGAACCTGGT-GCTGTCT-3'; M77349 forward, 5'-GCTGGAGAAATGGCATCATTATA-3', reverse, 5'-ACACCATGGCTCTGTACAATAG-3'; U81375 forward, 5'-TTTTCCATGTCCCCCTCCCAACT-3', reverse, 5'-AGGCACCTGGTTTC-TGTCAAATA-3'; U31511 forward, 5'-AGTGACCACCAGGAGGTGCTA-TC-3', reverse, 5'-AATCATACAGGGAAGACCCAAGT-3'; X75208 forward, 5'-ACAACCTCACGACAGTTGGTG-3', reverse, CTTATTGCCTT-TATTCTGTCTGTCG-3'; M33987 forward, 5'-CACAATTCGCGAGCCTTC-TATCA-3', reverse, 5'-TGTGTTCTTGAGGAAGGACAAGT-3'; M10617 forward, 5'-CTCTATTGCCACCATGAGTTTCT-3', reverse, 5'-AGTTCCG-GTCACAGACTTGATGTT-3'; U37518 forward, 5'-AGTGACCAACAT-AGTGAAACCCC-3', reverse, 5'-GAGTGTAGTGGCATGATCTACC-3'; AA524144 forward, 5'-TGTCTTTCAAACCCAAGGTTCC-3', reverse, 5'-TATACAAAAGTCCAAACAGCCAGG-3'; AF029082 forward, 5'-GTATTG-AGCAGAAAAGCAACGAG-3', reverse, 5'-TGAGTGTACAGGGGAAC-TTTAT-3'; control (HRS) forward, 5'-CAGCGGAAGACTGAAGAGTGG-3', reverse, 5'-GAGGCATTTTATTGGACCTTTG-3'.

Results

A set of microarrays containing 9216 human cDNAs in duplicate was hybridized with a mixture of Cy3-labeled cDNA probes corresponding to cancerous cells and Cy5-labeled cDNA probes corresponding to noncancerous epithelial cells. To identify genes with significantly different expression between carcinoma cells and noncancerous epithelial cells, rational Cy3/Cy5 cutoff values were determined (see “Material and Methods”). The number of genes categorized into these three groups and the number of genes under threshold signal intensities in each sample is summarized in Table 1.

Because advanced colorectal cancers show a diversity of expression profiles, we examined the ratios in all of the eight pairs of clinical materials. Genes with changed expression in more than half (5 of 8) cases were defined as the commonly up-regulated or down-regulated genes. As a result, 44 genes fell into the group of commonly up-regulated genes (Fig. 2A), whereas 191 genes fell into the group of commonly down-regulated genes (Fig. 2B). We also calculated *P* for each gene using a permutation test (see “Material and Methods”).

To examine the reliability of our microarray data, we selected five up-regulated genes (*TGFBI*, *IFITM1*, *SLC29A1*, *NOS2A*, and *EPHB3*)

⁵ <http://www.nhgri.nih.gov/DIR/LCG/ARRAY/expn.html>.

Table 1 The number of genes that showed increased, decreased, and unchanged expression

Sample no.	No. of genes			Intensity < 10 ⁵ (rfu) ^a	Total
	Increased	Decreased	Unchanged		
1	414	1013	438	7351	9216
2	261	939	609	7407	9216
3	34	693	452	8037	9216
4	270	641	459	7846	9216
5	37	252	1147	7780	9216
6	22	734	307	8153	9216
7	157	656	1540	6863	9216
8	137	909	482	7688	9216

^a rfu, relative fluorescent unit.

and five down-regulated genes (*CAI*, *MXII*, *SFN*, *FABP1*, and *TNFSF20*) and examined their expression levels by semiquantitative RT-PCR using the same aRNA samples that had served for the microarray analysis. The results of RT-PCR were consistent with microarray data in 64 of the 74 experiments. Therefore, we estimated the concordance between the microarray data and the RT-PCR data to be 86.5% (data not shown).

Furthermore, altered expression of these genes was tested using unamplified RNAs from an additional 12 cases (Fig. 3). The sample

pairs that showed tumor:normal PCR products ratio detected by densitometry of more than 1.5 or less than 0.67 were considered to be changed. The five up-regulated genes showed elevated expression in 10, 11, 10, 10, and 12 cancers of the 12 cases, respectively. On the other hand, the five down-regulated genes showed reduced expression in 10, 8, 8, 12, and 8 cancers of the 12 cases, respectively. These data verified the reliability and rationality of our strategy to identify genes that are commonly involved in the development and progression of colorectal cancer.

Discussion

To obtain exact information about differences in gene expression profiles between cells of normal colonic mucosa and colorectal cancer cells, one must have pure populations of each type of cell. LCM is a powerful way to extract desired cells selectively from tissue specimens. When combined with this technique, microarray analysis enabled effective identification of genes, the expression of which is (or can be) changed during colorectal carcinogenesis.

Among the genes listed in Fig. 2, A and B, differential expression of the transforming growth factor- β induced M_r 68,000 gene (*TGFBI*; GenBank accession no. M77349), the carbonic anhydrase I gene (*CAI*; GenBank accession no. M33987), the carbonic anhydrase II

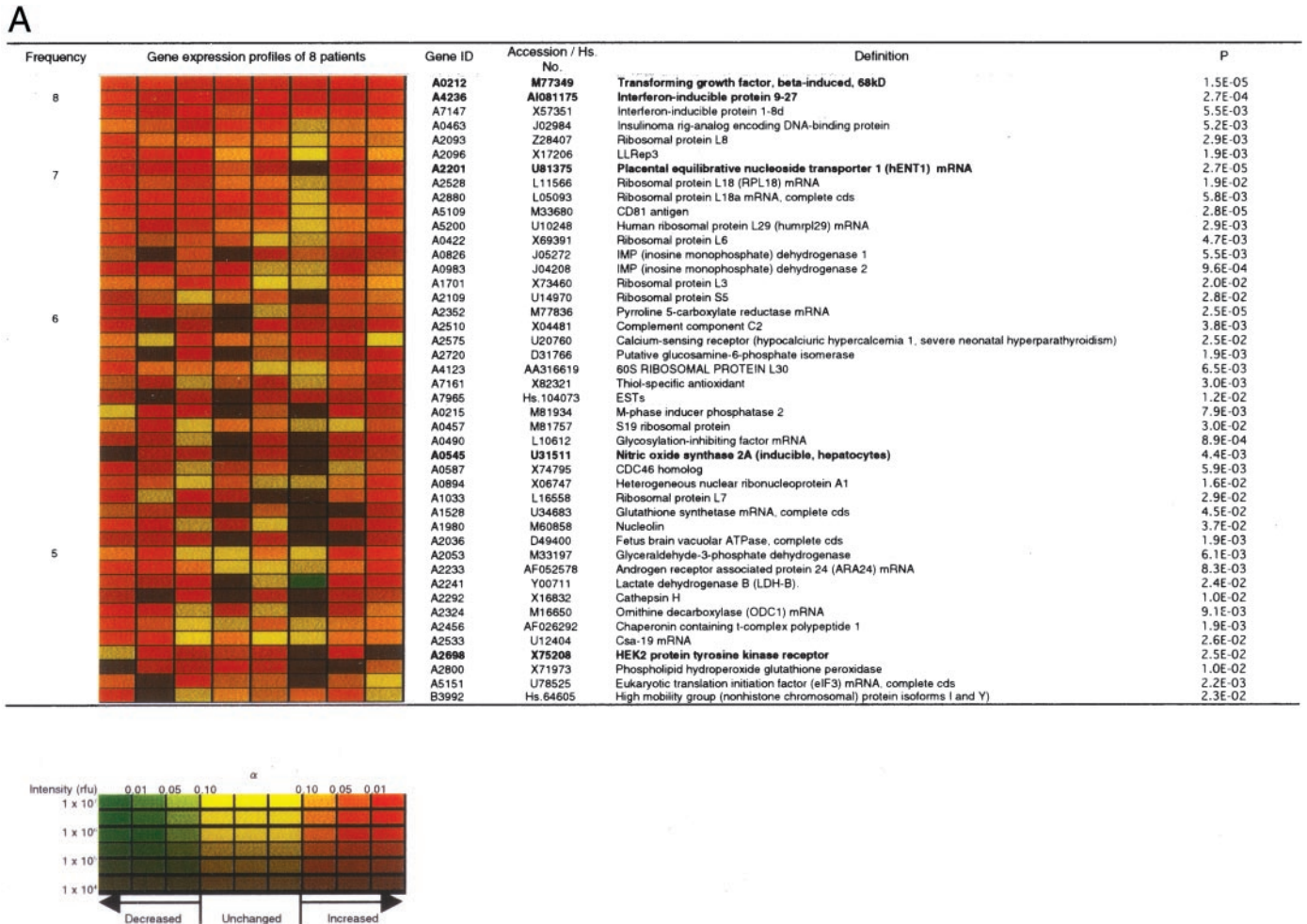


Fig. 2. A, representative list of up-regulated genes in colorectal carcinoma cells. The 44 genes showing up-regulated expression in five or more of eight tumors are listed. Genes in *bold type* were confirmed by RT-PCR. The numbers in the *frequency column* show the number of cases in eight patients. In the *bottom diagram*, α indicates significance level. *P* assigned with each gene was calculated using permutation test (as described in "Materials and Methods"). B, representative list of down-regulated genes in colorectal carcinoma cells. The 85 genes showing down-regulated expression in six or more of eight tumors are listed. Genes in *bold type* were confirmed by RT-PCR. The numbers in the *frequency column* show the number of cases in eight patients. In the *bottom diagram*, α indicates significance level. *P* assigned with each gene was calculated using permutation test (as described in "Materials and Methods").

gene (*CA2*; GenBank accession no. J03037), and the cytokeratin 20 gene (*KRT20*; GenBank accession no. X73502) had been noted before between normal colonic mucosa and colorectal cancers (9). Because *TGFBI*, isolated from a human lung-adenocarcinoma cell line A549

(22), has a recognition site for integrin, the gene product would represent a key molecule for adhesion and migration of malignant cells once a cancer has developed. The *CA1*, on the other hand, is expressed in normal colonic mucosa, but its reduced expression cor-

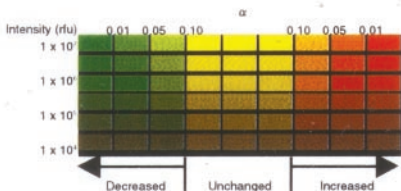
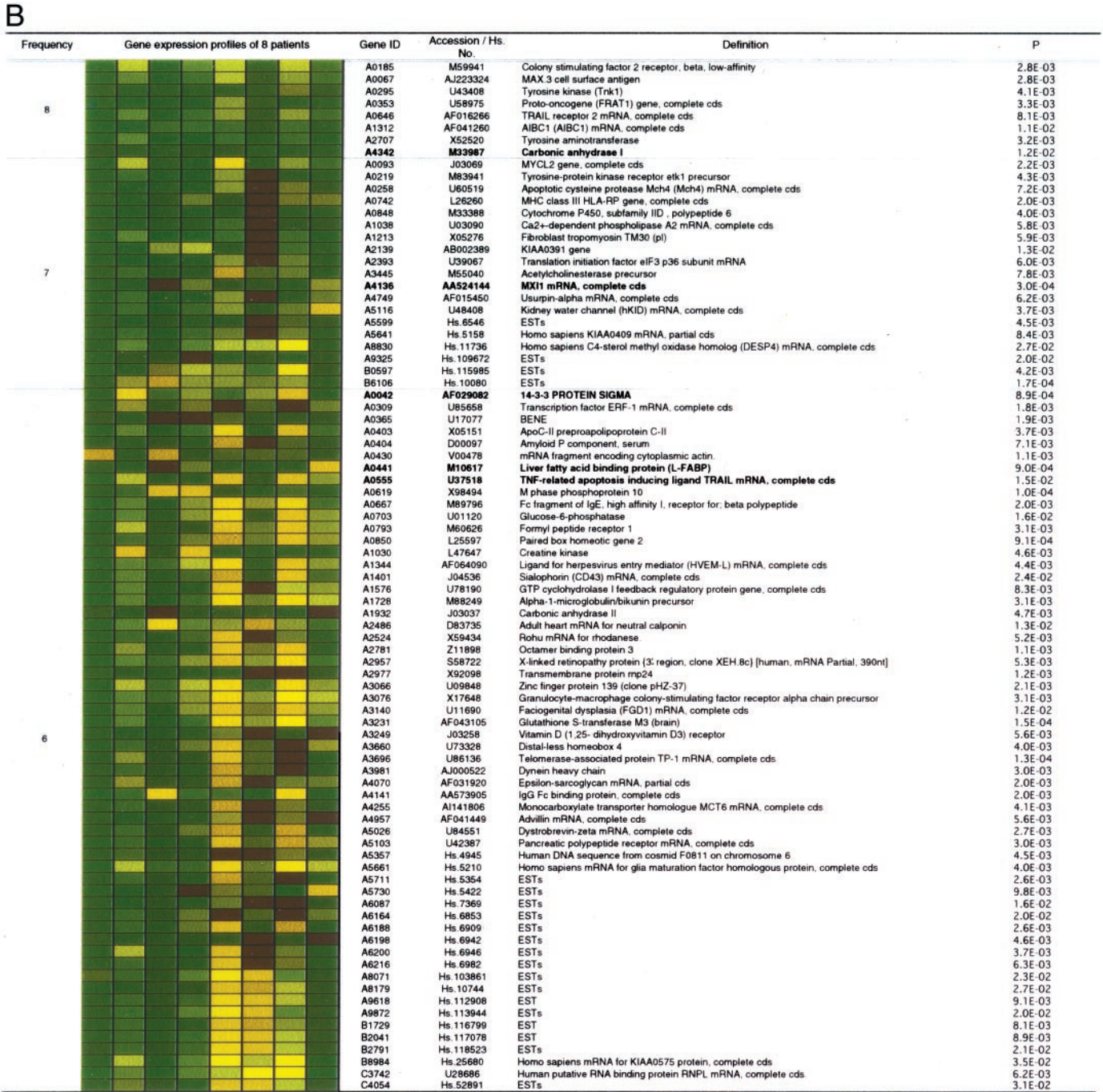


Fig. 2. Continued.

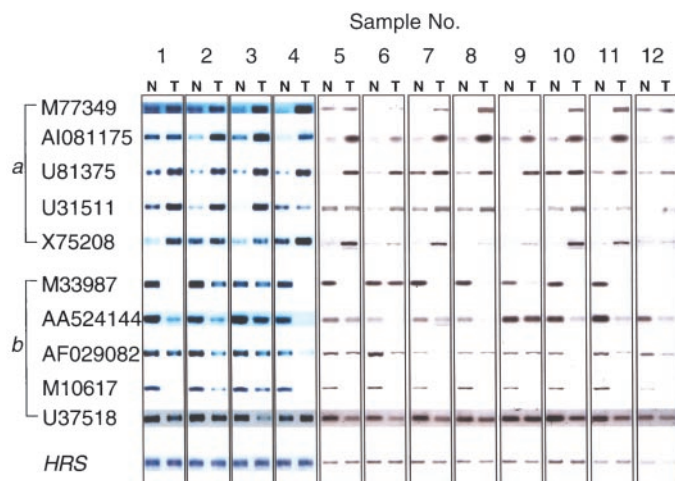


Fig. 3. Semiquantitative RT-PCR using RNAs from 12 newly obtained pairs of colorectal cancer cells and corresponding noncancerous colonic epithelial cells. *HRS* served as an internal control for each reaction. *a*, genes with up-regulated expression; *b*, genes with down-regulated expression; *N*, noncancerous colonic epithelial cells; *T*, colorectal cancer cells.

relates with vascular invasion and poor prognosis of colorectal cancers (23).

Other genes listed in Fig. 2B include 14-3-3 σ gene (*SFN*; AF029082), which is regulated by p53. 14-3-3 σ is known to promote premitotic cell-cycle arrest after DNA damage (24). Because this gene is one of the targets of p53, a gene that is frequently mutated in colorectal carcinomas, reduced expression of *SFN* in the cancer tissues studied here may have resulted from mutations of p53.

Among other genes in the list of those showing up-regulated expression in colorectal carcinoma cells, we note the presence of equilibrative nucleoside transporter1 (*ENT1*) gene (*SLC29A1*; U81375), M-phase inducer phosphatase 2 gene (*CDC25B*; M81934), nitric oxide synthase 2A gene (*NOS2A*; U31511), and nucleolin gene (*NCL*; M60858). In highly proliferative cancer cells, *ENT1* may help to supply nucleotides required for constitutive DNA synthesis (25). The *CDC25B* protein is required for progression of the cell cycle (G_2 -M phase transition) by dephosphorylating Tyr15 of cell division cycle 2 (*CDC2*) (26, 27). The transcript of *NOS2A*, when induced by various stimuli, causes production of nitric oxide, which may be involved in DNA damage as well as several kinds of signal transduction (28). Nucleolin induces chromatin decondensation by binding to histone H1 (29). The enhanced expression of these genes may have a direct or indirect role in the mechanisms of colorectal carcinogenesis including accelerated metabolisms, impaired regulation of cell cycle, production of reactive oxygen species, and dysregulated transcription and mitosis.

Genes showing down-regulated expression in colorectal carcinoma cells include the *MXI1* gene (*MXI1*; AA524144), a member of the c-Myc family, and several apoptosis-related genes: TRAIL receptor 2 gene (*TNFRSF10B*; AF016266), a receptor of TRAIL, TRAIL gene (*TNFSF10*; U37518), herpes virus entry mediator ligand gene (*TNFSF14*; AF064090), which are members of the tumor necrosis factor superfamily, and *Mch4* gene (*CASP10*; U60519), an activator of other caspases including caspase-3 and caspase-7 (30). The transcript of *MXI1* is a putative tumor suppressor (31), because it negatively regulates the action of c-Myc, an oncogene that activates transcription and stimulates cell proliferation. Therefore, inactivation or suppression of *MXI1* might promote malignant transformation and proliferation of affected cells. Because TRAIL receptor 2 induces apoptosis as a consequence of activation of nuclear factor- κ B and c-Jun-NH₂-terminal kinase (32), its reduced expression in colon can-

cers may prolong the survival of tumor cells. From the investigation of differently expressed genes in the hepatocarcinogenesis by microarray, we also found the expression of TRAIL receptor 2 was reduced in 10 hepatocellular carcinomas among 20 (data not shown). Therefore, escaping from the apoptosis signal may be a crucial step for carcinogenesis in various organs.

Our data provide not only new information about cancer-related genes but also a new correlation of known genes with carcinogenesis. We were even able to identify a relationship to cancer among some genes, the functions of which had not yet been characterized. These results not only disclose the complex nature of changes in genetic activity during colorectal carcinogenesis but also provide information that may prove useful for identifying novel therapeutic targets.

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