

Overexpression of the Aldo-Keto Reductase Family Protein AKR1B10 Is Highly Correlated with Smokers' Non-Small Cell Lung Carcinomas

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ABSTRACT

Purpose: Squamous cell carcinoma (SCC) and adenocarcinoma of the lung are currently subject to similar treatment regimens despite distinct differences in histology and epidemiology. The aim of this study is to identify a molecular target with diagnostic and therapeutic values for SCC.

Experimental Design: Genes specifically up-regulated in SCC were explored through microarray analysis of 5 SCCs, 5 adenocarcinomas, 10 small cell lung carcinomas, 27 normal tissues, and 40 cancer cell lines. Clinical usefulness of these genes was subsequently examined mainly by immunohistochemical analysis.

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Results: Seven genes, including aldo-keto reductase family 1, member B10 (*AKR1B10*), were identified as SCC-specific genes. *AKR1B10* was further examined by immunohistochemical analysis of 101 non-small cell lung carcinomas (NSCLC) and its overexpression was observed in 27 of 32 (84.4%) SCCs and 19 of 65 (29.2%) adenocarcinomas. Multiple regression analysis showed that smoking was an independent variable responsible for *AKR1B10* overexpression in NSCLCs ($P < 0.01$) and adenocarcinomas ($P < 0.01$). *AKR1B10* staining was occasionally observed even in squamous metaplasia, a precancerous lesion of SCC.

Conclusion: *AKR1B10* was overexpressed in most cases with SCC, which is closely associated with smoking, and many adenocarcinoma cases of smokers. These results suggest that *AKR1B10* is a potential diagnostic marker specific to smokers' NSCLCs and might be involved in tobacco-related carcinogenesis.

INTRODUCTION

Lung cancer is the leading cause of cancer death among all types of cancers and continues to increase in frequency worldwide (1). There are two major types of lung cancer, small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC), which account for 20% and 80% of all cases (2), respectively. NSCLC is further classified into squamous cell lung carcinoma (SCC) and lung adenocarcinoma. Despite distinct differences in histologic and epidemiologic features, adenocarcinoma and SCC are similarly treated in clinical practice (3) partly because underlying molecular mechanisms are largely unknown. Even the most recent therapeutic innovations for NSCLC have yielded little improvement to prognosis with overall 5-year survival rates still <15% (4).

We reported previously the clinical relevance of expression of G₁-S transition regulatory molecules in prognosis, such as p53, retinoblastoma protein, p16^{INK4A}, and p27 in NSCLCs (5–8). We further showed that Ki-67-positive, high-level cyclin E, low-level *N*-acetylgalactosaminyl transferase-3 (GalNAcT3) and low-level *N*-acetylglucosaminyltransferase (GnT-V) are associated with shorter survival in NSCLCs (8–12). However, we did not observe any differences between SCC and adenocarcinoma.

SCC accumulates a series of genetic alterations in the progression from a normal bronchial epithelium, metaplasia, dysplasia, and carcinoma *in situ* to invasive carcinoma (13). Because most SCC develops in smokers and tobacco smoking reversibly induces metaplasia, smoking has been regarded as a major cause of SCCs (14). As diagnostic markers for SCC, SCC antigen and cytokeratin 19 fragment (CYFRA 21.1) have been widely used (15). Despite their usefulness in distinguishing between SCC and adenocarcinoma, these two molecules are hardly adequate for early detection of cancer (15). Moreover, their expression in normal squamous cell suggests that these two

molecules are not involved in carcinogenesis and inappropriate as therapeutic targets. Thus, search for genes specific to SCC alone will lead to identification of a novel molecular target of SCC, which may help developing both early detection of SCC and personalized therapeutics of SCC.

Microarray analysis has been applied to several aspects of cancer research, including classification, mechanistic elucidation, discovery of therapeutic targets, and development of tumor makers (16–21). For example, we recently explored potential diagnostic or therapeutic markers of hepatocellular carcinoma using microarray analysis and showed that soluble glypican-3 is a novel serologic marker essential for early detection of hepatocellular carcinoma (19). Recent reports on microarray analysis of lung cancer have shown that SCC and adenocarcinoma have different gene expression signatures, suggesting involvement of distinct pathways in carcinogenesis (22, 23). In the present study, we searched for genes specifically overexpressed in SCC through microarray analysis and identified seven genes, including aldo-keto reductase family 1, member B10 (*AKR1B10*). We investigated potential relevance of *AKR1B10* in NSCLCs with a newly generated monoclonal antibody and found that it is overexpressed in smokers' NSCLCs, including most cases with SCC.

MATERIALS AND METHODS

Tissue Samples and Cell Lines. Forty-five primary lung cancers (15 SCCs, 20 adenocarcinomas, and 10 SCLCs) were obtained with informed consent from patients who underwent lobectomy at Jichi Medical School Hospital (Tochigi, Japan), Cancer Institute Hospital, Japanese Foundation for Cancer Research (Tokyo, Japan), and Hokkaido University Medical Hospital (Hokkaido, Japan). All samples were immediately frozen after resection and stored at -80°C until RNA or protein was extracted. Adenocarcinoma cell lines A549, H23, H522, H1648, and H2347 were purchased from the American Type Culture Collection (Manassas, VA). SCLC cell line Lu130 and SCC cell line H157 were obtained from Cell Resource Center for Biomedical Research, Tohoku University (Miyagi, Japan).

RNA Extraction and Microarray Analysis. Tissues or cells were directly lysed in Isogen reagent (Nippon Gene, Osaka, Japan) and homogenized. Total RNA was extracted according to manufacturer's instructions. Surgically resected lung tissues and lung cancers, including 5 SCCs, 10 SCLCs, a pooled sample made up of 12 adenocarcinomas, other 5 adenocarcinomas, and 1 normal lung, were analyzed on GeneChip HG U133 oligonucleotide arrays (Affymetrix, Santa Clara, CA) containing probes for ~40,000 human genes. Further information on the source of

other RNA from normal tissues analyzed here is provided on request or is available at <http://www.lsbm.org/db/index.html>. Microarray analysis was done essentially as described previously (24). For global normalization, the average signal in an array was made equal to 100.

Systematic Selection of SCC-Specific Genes Based on Microarray Analysis. We systematically explored SCC-specific genes that were defined as follows: its expression level is (a) up-regulated in SCC but minimal in (b) normal lung and bronchial epithelia, (c) adenocarcinoma and SCLC, and (d) normal squamous epithelia, such as skin. Briefly, genes with a median signal score across 5 SCCs of >150 and >10 times that of normal lung were first selected. Among the 136 genes selected, genes with signal score of >150 in skin and small airway epithelial cell were omitted. We subsequently eliminated genes with signal >150 in SCLCs, adenocarcinomas, and most other normal tissues and various primary culture cells. Among 12 genes selected thus far, we additionally eliminated 5 genes that showed low expression throughout all of 40 cancer cell lines, suggesting expression by surrounding stromal cells but not by cancer cells (Table 1).

Quantitative Real-time Reverse Transcription-PCR. After digesting genomic DNA using DNase I (Invitrogen, Carlsbad, CA) cDNA was synthesized from 1 μg total RNA using SuperScript First-Strand Synthesis System for reverse transcription-PCR (Invitrogen) in 24 μL volume and diluted up to 80 μL . Quantitative real-time PCR for *AKR1B10* were done using an iCycler iQ Detection System (Bio-Rad, Hercules, CA). Reaction mixtures contained SYBR Green I nucleic acid gel stain (BMA, Rockland, ME) and primers 5'-CCCAAAGATGATAAAGGTAATGCCATCGGT-3' and 5'-CGATCTGGAAGTGGCTGAAATTGGAGA-3' for *AKR1B10* or 5'-AGAAGGAGATCACTGCCCTGGACC-3' and 5'-CCTGCTTGCTGATCCACATCTGCTG-3' for β -actin. PCR condition was 1 cycle of 94°C for 3 minutes followed by 40 cycles at 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 1 minute. All the samples were run in triplicate, and the results were averaged. Specific amplification of *AKR1B10* was confirmed by the gel electrophoresis and melting curve analysis after PCR. The expression level of *AKR1B10* was indicated as a relative ratio of its signal to that of β -actin to normalize the starting amount of template cDNA. We also did semiquantitative PCR using the same condition for six pair-samples of SCC and corresponding noncancerous lung tissues.

Generation of Anti-*AKR1B10* Monoclonal Antibodies. Monoclonal antibodies against *AKR1B10* were generated as described previously (25). Briefly, glutathione *S*-transferase–fused

Table 1 Genes with SCC-specific overexpression

Symbol	Gene name	UniGene no.	Functional classification
<i>AKR1B10</i>	Aldo-keto reductase family 1, member B10	Hs.116724	Retinal reductase
<i>ELAFIN</i>	Elafin	Hs.112341	Protease inhibitor
<i>AKR1C1</i>	Aldo-keto reductase family 1, member C1	Hs.431175	Detoxification
<i>SPRR3</i>	Small proline-rich protein 3	Hs.139322	Structural component
<i>ALDH3A1</i>	Aldehyde dehydrogenase 3 family, member A1	Hs.575	Detoxification
<i>NQO1</i>	NAD(P)H dehydrogenase, quinone 1	Hs.406515	Activation of carcinogens
<i>UGT1A9</i>	UDP glycosyltransferase 1 family, polypeptide A9	Hs.375197	Detoxification

NOTE. Final seven genes selected through microarray analysis are listed. Representative function of each gene is summarized from the literature or National Center for Biotechnology Information Web site (<http://www.ncbi.nlm.nih.gov>).

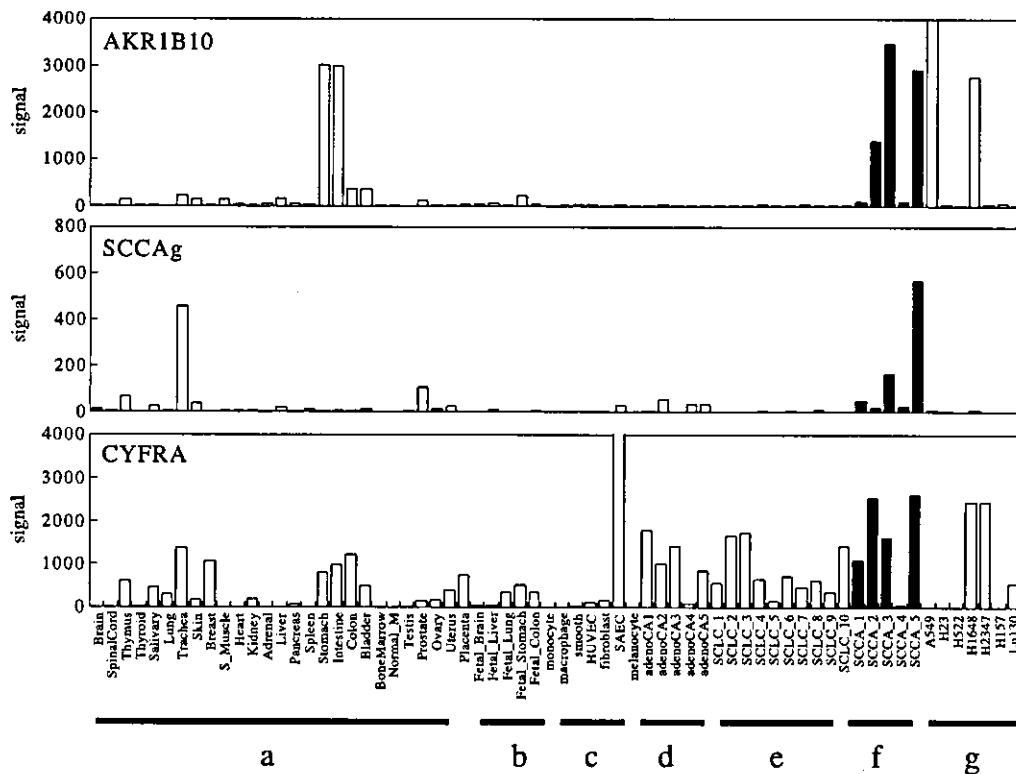


Fig. 1 Expression profiles of AKR1B10. A tissue-wide expression of AKR1B10 was displayed with CYFRA and SCCAg as references. Signal denotes gene expression level obtained from microarray analysis: (a) 27 normal tissues, (b) 5 fetal tissues, (c) 7 cultured normal cells, (d) 5 adenocarcinomas, (e) 10 SCLCs, (f) 5 SCCs, and (g) 7 lung cancer cell lines. Filled columns, SCC.

full-length AKR1B10 produced in *Escherichia coli* was immunized to female BALB/c mice. Nine clones of monoclonal hybridomas were selected by immunoblotting against recombinant AKR1B10 transiently expressed in COS-7 cells. We selected H4025 as a specific antibody in this study because a single band at around M_r 36,000 was observed only in AKR1B10-expressing cell lines as revealed by microarray analysis of 37 cell lines.

Immunoblot Analysis. Immunoblot analysis was done as described previously (25). Briefly, cells or tissues were lysed by 10 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 5 mmol/L EDTA, 1.0% Triton X-100, 1.0% sodium deoxycholate, 0.1% SDS with protease inhibitor cocktail (Sigma, St. Louis, MO) at 4°C. H4025 (5 µg/mL) or anti- β -actin antibody (0.3 µg/mL, Sigma) was used as primary antibodies.

Immunocytochemistry and Confocal Microscopy Analysis. Immunostaining of culture cells were done after fixation in 4% paraformaldehyde and permeabilization in 0.2% Triton X-100 followed by incubation with 2% nonfat milk in TBS. An antibody H4025 (50 µg/mL) was applied as a primary antibody and incubated in a moist chamber at room temperature for 1 hour. The secondary staining was done with FITC-labeled anti-mouse IgG antibody (Sigma) as secondary antibody at room temperature for 1 hour. Dual-color detection by confocal laser scan microscopy (TCS SP2 system, Leica, Bensheim, Germany) was done after treatment with a 0.5 µmol/L solution of the mitochondrial stain MitoTracker Red CMXRos (Invitrogen) or the intercalator of double-strand nucleic acid stain propidium iodide (Invitrogen).

Immunostaining Analysis. Immunohistochemical analysis for AKR1B10 was done with the formalin-fixed, paraffin-

embedded tissue archive at the University of Tokyo. The sections were deparaffinized in xylene, washed in ethanol, and rehydrated in TBS. Antigen retrieval was done in 10 mmol/L citrate buffer (pH 6.0) at 120°C for 10 minutes following incubation with TBS with 2% nonfat dried milk. Then, H4025 (50 µg/mL) or cytokeratin 5/6 (1:500, DAKO Ltd., Cambridge, United Kingdom) was applied for 1 hour followed by the secondary staining with DAKO Envision+ reagent. All sections were counterstained with Mayer's hematoxylin. We defined AKR1B10 positive if >10% of tumor cells displayed immunoreactivity.

We first examined archival samples of the University of Tokyo to compare expression of AKR1B10 and that of keratin 5/6 in NSCLCs, squamous epithelia of skin and esophagus, alveolar epithelium, and bronchus. We have analyzed previously 217 primary NSCLC specimens for expression of cyclin E, Ki-67, Bcl-2, p53, retinoblastoma protein, p27, GalNAcT3, and GnT-V (8, 9, 11, 12). Among these, we next examined 101 NSCLCs, which were classified into 32 SCCs, 65 adenocarcinomas, and 4 adenosquamous cell carcinomas according to WHO criteria (26). Clinicopathologic features are summarized in Table 3. The postsurgical pathologic tumor-node-metastasis stage was determined according to the guidelines of the American Joint Committee on Cancer (27). The Medical Ethical Committee of Hokkaido University School of Medicine approved this immunohistochemical study.

Statistical Analysis. We analyzed the statistical significance of the relationship between the expression of AKR1B10 and clinicopathologic variables by χ^2 test, Yates χ^2 test, or Fisher's exact test as appropriate. We also analyzed the associations between AKR1B10 expressions and the cyclin E or Ki-67 labeling index (%; refs. 8, 9) by Student's *t* test.

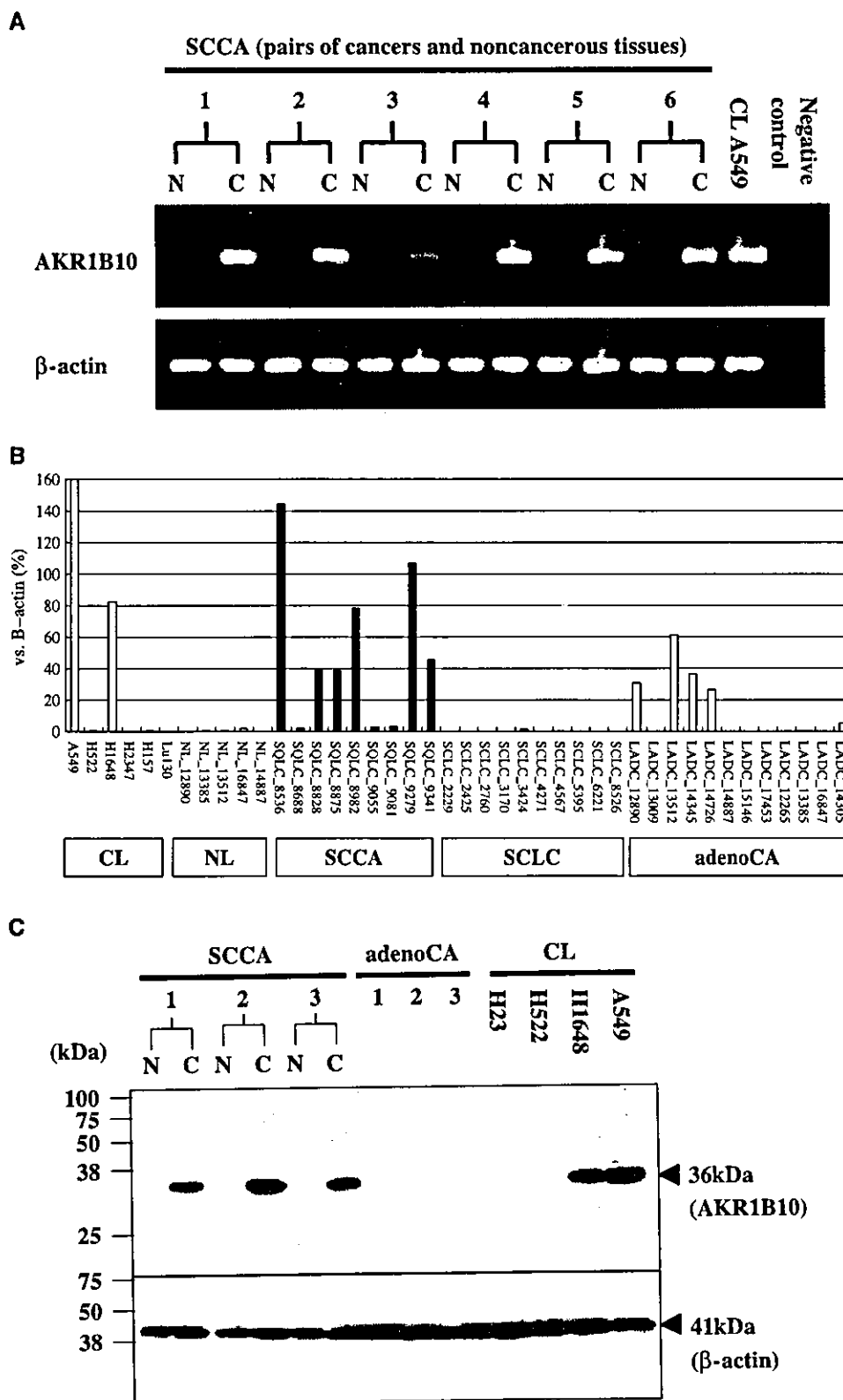


Fig. 2 Overexpression of AKR1B10 in SCC. *A*, semiquantitative PCR using six pairs of SCC and noncancerous lung tissues. Note that *AKR1B10* was up-regulated in all paired samples. *B*, quantitative real-time PCR. Examined samples were 9 SCCs, 12 adenocarcinomas, 10 SCLCs, 6 lung cancer cell lines, and 5 normal lung tissues. Note expression level of *AKR1B10* was remarkably high in 6 SCCs compared with 4 adenocarcinomas. *C*, immunoblot analysis of AKR1B10. A 36-kDa protein was detected in SCCs and AKR1B10-expressing cell lines H1648 and A549. *N*, normal lung tissues; *C*, cancer tissues; *CL*, cell line.

We additionally used multiple regression analysis to extract factors responsible for AKR1B10 expression in NSCLCs and adenocarcinomas alone. Sex, age, smoking history, differentiation, pT classification, pN classification, survival time, histology, cyclin E, Ki-67, GalNAcT3, and GnT-V were used as independent variables and AKR1B10 expression as a dependent variable. Differences were considered significant at $P < 0.05$. We simultaneously examined the correlation coefficient and the partial correlation coefficient between AKR1B10 expression and smoking or sex.

RESULTS

Microarray Analysis Identifies Seven Genes Specifically Up-Regulated in SCC. We selected seven potential SCC-specific genes (see Materials and Methods) using microarray analysis (Table 1). Tissue-wide expression profiles of these genes showed their high specificity compared with two widely used diagnostic markers of SCC, SCC antigen and CYFRA 21.1, suggesting robustness of our selection for SCC-specific genes (Fig. 1; Supplementary Fig. 1). Among these seven genes, *AKR1C1*, *ELAFIN*, *NQO1*, and *UGT1A9* were reported previously as potential target genes for detection or therapy against lung cancer (28–31); *SPRR3* is overexpressed in epidermal SCC (32); and *ALDH3A1* was reported to be involved in metabolism of tobacco carcinogens (33). Overexpression of *AKR1B10* has not been reported previously in lung cancer; then, we investigated whether it represents a good molecular target of SCC.

Overexpression of AKR1B10 in SCC. To confirm array data, we first did semiquantitative PCR. Overexpression of *AKR1B10* in SCC was observed in six pair-samples analysis (Fig. 2A). We subsequently investigated expression level of *AKR1B10* across lung cancer tissues by quantitative reverse transcription-PCR. Overexpression of *AKR1B10* was observed in 6 of 9 (67%) SCCs but not in SCLCs or normal lung tissues (Fig. 2B). Expression of *AKR1B10* was also observed in 4 of 12 (33%) adenocarcinomas, although its level was not so high as in SCC (Fig. 2B).

Next, we investigated expression of AKR1B10 protein by immunoblot analysis with a newly generated monoclonal anti-AKR1B10 antibody, H4025. As for three pair-samples of SCC used in semiquantitative PCR above, AKR1B10 was observed only in cancerous tissues, whereas AKR1B10 was negative in 3 adenocarcinomas (Fig. 2C).

Comparison of AKR1B10 with Pan-Squamous Cell Marker Keratin 5/6. As a SCC marker, keratin 5/6 is widely used based on its specificity to squamous cells. Unique feature of AKR1B10 as we identified in our selection is that it is not a merely squamous cell-specific marker unlike keratin 5/6 but a SCC-specific marker. To highlight the difference in “specificity” of these two molecules, we compared their expression in NSCLCs and normal tissues, including squamous epithelia of skin and esophagus, alveolar epithelium, and columnar epithelia of bronchus (Table 2). Keratin 5/6 staining was observed in normal squamous epithelia, columnar epithelia, and 83% of SCCs but not in adenocarcinoma. In contrast, AKR1B10 staining was observed in 64% of SCC and 30% of adenocarcinoma but not in normal epithelia (Table 2).

Distinct Localization of AKR1B10 in SCC and Adenocarcinoma. As we described above, analysis in larger

Table 2 Expression of AKR1B10 and keratin 5/6 in NSCLCs and normal epithelia

		AKR1B10	Keratin 5/6
NSCLCs	SCC ($n = 23$)	Positive (61%)	Positive (83%)
	Adenocarcinoma ($n = 24$)	Positive (33%)	Negative (0%)
Normal epithelia	Pulmonary alveoli ($n = 3$)	Negative	Negative
	Bronchial epithelia ($n = 3$)	Negative	Positive
	Squamous epithelia		
	Skin ($n = 3$)	Negative	Positive
	Esophagus ($n = 3$)	Negative	Positive

number of samples revealed that AKR1B10 was expressed not only in most cases of SCC but also in a subset of adenocarcinoma. To investigate relevance of AKR1B10 in NSCLCs, we subsequently did immunohistochemical analysis in 101 primary NSCLCs, including 65 adenocarcinoma (Table 3). AKR1B10 staining was observed in 27 of 32 (84.4%) SCCs but also in 19 of 65 (29.2%) adenocarcinomas (Table 3). In adenosquamous cell carcinomas, AKR1B10 staining was observed in 2 of 4 cases and restricted to SCC components of these 2 cases (data not shown). AKR1B10 was preferentially observed in cancer cells with obvious squamous differentiation in SCC (Fig. 3A–E), whereas with lower differentiation grade in adenocarcinoma (Fig. 3G). Interestingly, we occasionally observed AKR1B10 staining in lesions with metaplasia: squamous metaplasia (Fig. 3I) and transitional cell metaplasia (Fig. 3J) in noncancerous areas (Fig. 3K) of one smoker's SCC specimen. We seldom detected positive staining in noncancerous portion of lung tissue, except two cases in normal bronchial epithelia of smokers (Supplementary Fig. 2A and B).

AKR1B10 staining was mainly observed in cytoplasm of cancer cells but also in nucleus in a subset of cells (Fig. 3E and F). Notably, two cases had apparent AKR1B10 staining mainly in nuclei (Fig. 3M). These results were essentially confirmed in confocal microscopy analysis of A549 cells. AKR1B10 was generally localized in cytoplasm, neither in nucleus nor in mitochondria in most cells. However, a subset of cells had additional staining in nucleus in 70% confluency (Fig. 3L and M) but not in full confluency (Fig. 3N).

Correlation between AKR1B10 Overexpression and Smoking History in NSCLC and Adenocarcinoma. To clarify the factors that correlate with AKR1B10 immunostaining, we carried out a statistical analysis that examined a variety of clinicopathologic variables and the expression of molecules that we reported previously (refs. 8, 9, 11, 12; Table 3). We observed positive correlations between AKR1B10 overexpression and SCCs (χ^2 test, $P < 0.0001$) and smoking (χ^2 test, $P < 0.0001$) in NSCLCs. AKR1B10 overexpression was observed in 40 of 61 (65.6%) smokers' NSCLCs. The correlation coefficient between AKR1B10 overexpression and smoking was 0.47 in NSCLCs. Partial correlation coefficient was 0.41 even after removing the effect of positive correlation between AKR1B10 overexpression and male ($P < 0.05$). These results indicate the significant correlation between AKR1B10 overexpression and smoking.

Univariate analysis in NSCLCs also showed that AKR1B10 was overexpressed in tumors with high pT classification ($P < 0.05$). Additionally, AKR1B10-positive cases had a higher Ki-67 expression ($P < 0.001$), higher cyclin E expression ($P < 0.01$), lower GalNAcT3 expression ($P < 0.01$), and lower GnT-V expression ($P < 0.05$) than negative cases in NSCLCs. Student's *t* test revealed that there was a significant difference between AKR1B10 expression and Ki-67 expression ($P < 0.005$) and cyclin E expression ($P < 0.05$) in NSCLCs.

Multiple regression analysis showed that smoking ($P < 0.01$), SCC ($P < 0.01$), and lower GalNAcT3 ($P < 0.05$) were important independent variables responsible for AKR1B10 overexpression in NSCLCs (Table 4). We subsequently analyzed only adenocarcinomas ($n = 65$) because most SCCs were AKR1B10 positive (84.4%) and smokers (96.9%). Interestingly, there was still a remarkable correlation (χ^2 test, $P < 0.01$) between AKR1B10 overexpression and smoking in adenocarcinomas (Table 3). Moreover, it was also shown that smoking was the only important independent variable responsible for AKR1B10 expression in adenocarcinomas ($P < 0.01$; Table 4).

DISCUSSION

Aldo-keto reductases are NAD(P)H-dependent oxidoreductases that catalyze the reduction of a variety of carbonyl compounds (34). AKR1B10 is a member of this superfamily and reduces aromatic and aliphatic aldehyde substrates (34). Reportedly, AKR1B10 mRNA shows expression in adrenal gland, small intestine, and colon, consistent with its putative physiologic roles in steroid metabolism or detoxification of reactive aldehydes in the digested food in intestinal tract (34–36).

Initial goal of our study was to identify SCC-specific molecules, distinct from currently used SCC markers that are specific to squamous cell in general. We eliminated these squamous cell marker genes through our selection and identified *AKR1B10* as a gene highly specific to SCC but not to squamous cells in general. AKR1B10 was expressed in as many as 90% of SCC of the lung but not in normal bronchial epithelium and squamous epithelium from skin and esophagus. This unique feature of AKR1B10 is highlighted when we compared the results of immunohistochemical analysis using AKR1B10 and keratin 5/6 (Table 2). AKR1B10 was highly specific to SCC when SCC and normal epithelia were analyzed by immunohistochemistry, although its specificity and sensitivity for SCC among NSCLCs were lower than those of keratin 5/6.

In the present study, we showed that AKR1B10 is overexpressed in SCC, which is closely associated with smoking. Additionally, we found AKR1B10 expression even in metaplasia, which is also associated with smoking and regarded as precancerous lesions of SCC (37, 38). Unexpectedly, nearly one third of the cases of adenocarcinomas expressed AKR1B10, but it was revealed by multiple regression analysis that smoking was the most important determinant of AKR1B10 expression in adenocarcinomas. Adenocarcinomas can be clustered into several subclasses based on reported expression profiling (22, 23). Together with recent reports that ~40% of adenocarcinomas occur in

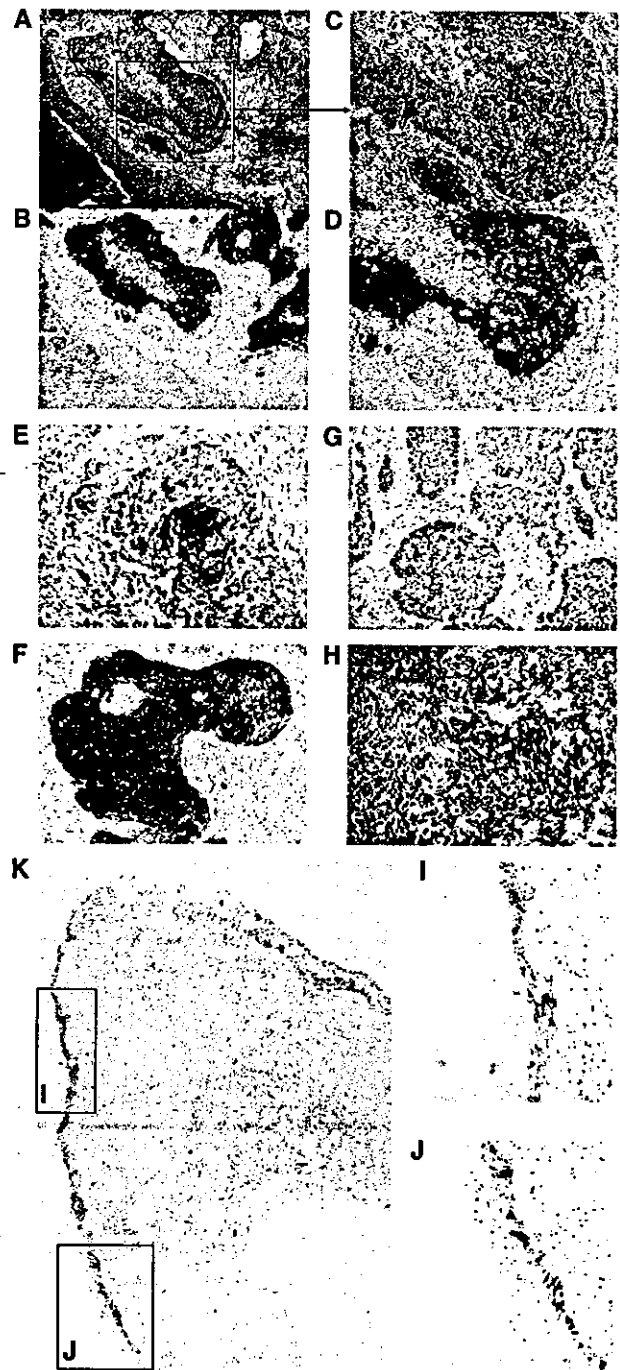


Fig. 3 Immunohistochemical analysis of AKR1B10. *A-E*, two representative cases in SCC. H&E staining, (*A*) $\times 20$ and (*C*) $\times 100$. Cancerous regions with obvious (red line) and no (blue line) squamous differentiation. Corresponding staining of the same sample (*B* and *D*) and another sample (*E*) by H4025. Note that AKR1B10 is stained in regions with squamous differentiation. *F* and *G*, two representative cases in adenocarcinoma. Homogenous staining was observed in some cases (*F*), whereas preferential staining in regions with lower differentiation was observed in most cases (*G*). *H*, typical case with nuclear staining in SCC ($\times 100$). *I-K*, AKR1B10 staining in metaplasia of a smoker. *I*, squamous metaplasia: (*left*) $\times 20$ and (*right*) $\times 100$. *J*, transitional cell metaplasia: (*left*) $\times 20$ and (*right*) $\times 100$. Note that these metaplastic regions are observed successively in noncancerous regions of a case with SCC (*K*).

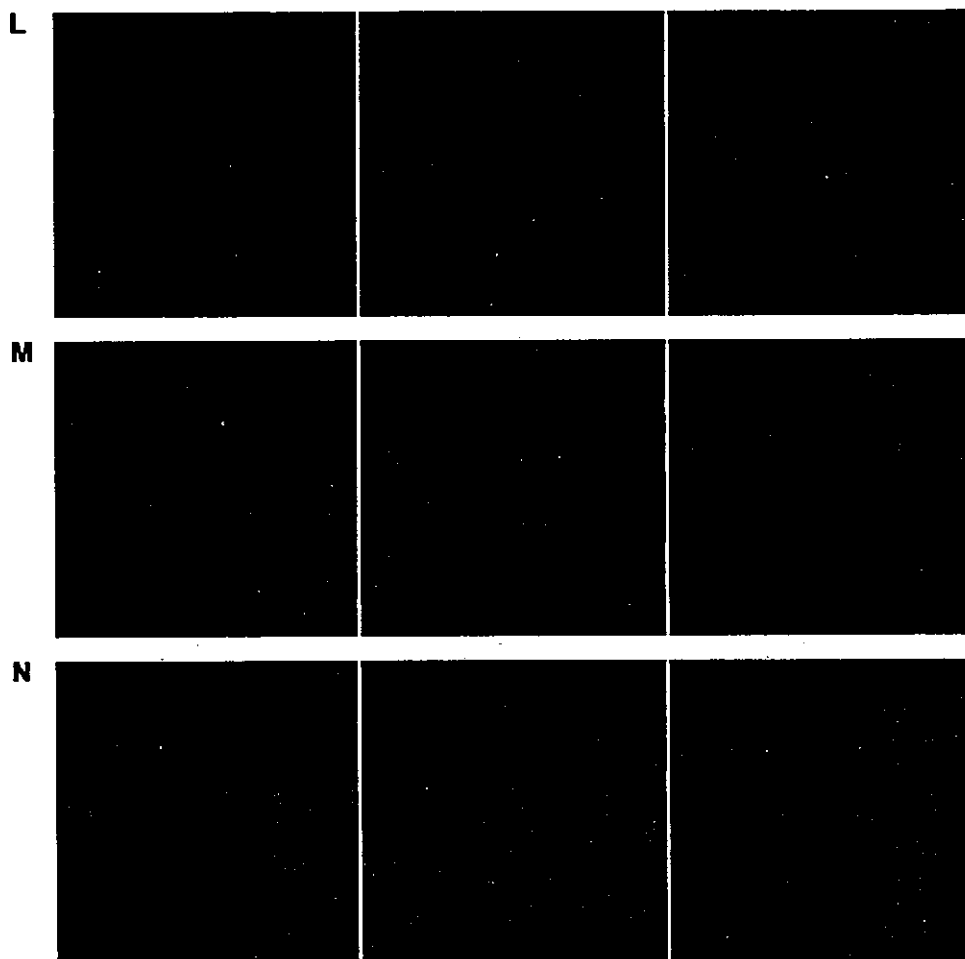


Fig. 3 Continued L-N, subcellular localization of endogenous AKR1B10 in A549 cells in 70% (L and M) and 100% (N) confluency. Left, AKR1B10; middle, MitoTracker (L) or propidium iodide (M and N); right, merged image. Note nuclear staining (L and M) has disappeared in 100% confluency (N).

smokers (39), there is a possibility that AKR1B10 could characterize a subset of adenocarcinoma associated with smoking. Based on our results, AKR1B10 immunostaining could be applied to the early detection of cancer cells or atypical cells in sputum, especially in heavy smokers.

Then, what could be potential roles of AKR1B10 in multistep carcinogenesis of SCCs? There are two possibilities as follows: one is that AKR1B10 may be related to cell proliferation. There was a positive correlation between AKR1B10 expression and putative poor prognosis factors, such as high Ki-67, high cyclin E, low GalNAcT3, and low GnT-V in NSCLCs (8, 9, 11, 12). Moreover, AKR1B10 was localized in nucleus in a fraction of cancer cells in subconfluent culture conditions, which disappeared under confluent culture, suggesting that AKR1B10 translocates during cell cycle and is involved in the regulation of cell cycle in a fashion yet identified.

Another possibility is that AKR1B10 promotes carcinogenesis of SCC through its enzymatic activity that counteracts the conversion of β -carotene to retinoic acid (40). Retinoic acid induces potent differentiation and growth-suppressive effects in diverse premalignant and malignant cells (41). In lung, deficiencies of retinoids are reported to cause hyperplasia and squamous metaplasia of airway epithelium (42) that can be

suppressed by retinoic acid (43). Through the analysis of many cancer samples, we noticed positive staining of AKR1B10 even in some cases with metaplasia, precancerous lesion of SCC. Because the number of samples that contained metaplasia was small in the present study, this result was further investigated by another study focusing on idiopathic pulmonary fibrosis, which showed that squamous metaplasia was positive for AKR1B10 in 23 cases of 56 squamous metaplasia lesions.¹⁰ These results strongly suggest that AKR1B10 expression is positive in precancerous lesions and may down-regulate retinoic acid, which could lead to carcinogenesis of SCC. Considering that AKR1B10 is an enzyme related to detoxification and that some smokers' bronchial epithelia without metaplasia were positive for AKR1B10 staining, AKR1B10 may be directly induced by some chemical compounds in tobacco, which should be further investigated. Interestingly, we also observed frequent overexpression of AKR1B10 in SCC of the laryngopharynx and esophagus that is closely associated with smoking and occasional overexpression of esophageal dysplasia and

¹⁰ Fukayama et al., in preparation.

hyperplasia.¹⁰ Remarkably high frequency of its up-regulation specific to SCC warrants further investigation of AKR1B10 in carcinogenesis of SCC.

Various retinoids, including β -carotene, have been shown previously effective for the treatment and prevention of several cancers, including carcinoma of the breast, skin, and kidney (44–49). However, clinical chemoprevention trials of lung cancer by β -carotene have failed to show its effectiveness. Moreover, administration of β -carotene unexpectedly promoted tumorigenesis in smokers (50, 51). Molecular mechanism

underlying these adverse effects is currently unknown, but up-regulation of AKR1B10 in precancerous lesions in the bronchial epithelium of smokers may partly explain ineffectiveness of β -carotene observed in the lung.

AKR1B10 was also overexpressed in adenocarcinoma of smokers. Its staining was observed in undifferentiated region in contrast to SCC with staining in differentiated region. Together with its overexpression in hepatocellular carcinomas (34, 36), AKR1B10 may be related to another carcinogenic pathway distinct from that of SCC.

Table 3 Clinicopathologic features correlated to AKR1B10 overexpression

Characteristics	NSCLC	AKR1B10		P, χ^2 test	Adenocarcinoma	AKR1B10		P, χ^2 test or Fisher test
		Positive	Negative			Positive	Negative	
Age (y)								
Median (range)	63 (31-85)							
<65	58	28	30	NS	37	9	28	NS
\geq 65	43	20	23		28	10	18	
Sex								
Male	63	36	27	<0.05	30	10	20	NS
Female	38	12	26		35	9	26	
Histology								
Squamous	32	27	5	<0.0001				
Adenocarcinoma	65	19	46					
Adenosquamous	4	2	2					
Differentiation (SCC)								
Poor	14	9	5	<0.05	10	2	8	NS
Moderate/well	15	15	0		49	15	34	
Smoking								
Smoker	61	40	21	<0.0001	30	14	16	<0.01
Nonsmoker	36	6	30		35	5	30	
pT classification								
T ₁	33	11	22	<0.05	21	4	17	NS
T ₂ -T ₃	68	37	31		44	15	29	
pN classification								
N ₀	56	28	28	NS	36	13	23	NS
N ₁ -N ₃	45	20	25		29	6	23	
Ki-67								
High labeling index*	51	33	18	<0.001	22	11	11	<0.05
Low labeling index†	48	14	34		41	7	34	
Cyclin E								
Positive	76	42	34	<0.01	41	13	28	NS
Negative	24	5	19		23	5	18	
Bcl-2								
Positive	14	8	6	NS	6	2	4	NS
Negative	29	9	20		22	3	19	
p27								
Positive	87	42	45	NS	53	14	39	NS
Negative	9	4	5		8	3	5	
p53								
Positive	28	13	15	NS	16	4	12	NS
Negative	17	5	12		13	1	12	
Retinoblastoma protein								
Positive	31	15	16	NS	18	5	13	NS
Negative	12	2	10		11	1	10	
GalNAcT3								
Positive	63	21	42	<0.01	49	11	38	NS
Negative	34	24	10		14	7	7	
GnT-V								
High‡	47	17	30	<0.05	39	10	29	NS
Low§	52	31	21		24	9	15	

NOTE. NS, not significant.

* \geq 30% of cancer cells stained.

†<30% of cancer cells stained.

‡50% of cancer cells stained.

§<50% of cancer cells stained.

Table 4 Multiple regression analysis for AKR1B10

Characteristics	NSCLCs			Adenocarcinomas		
	Regression coefficient	P	95% Confidence interval	Regression coefficient	P	95% Confidence interval
Age	0	0.432	-0.15 to 0.006	-0.01	0.34	-0.02 to 0.007
Sex	-0.19	0.191	-0.474 to 0.096	-0.23	0.13	-0.534 to 0.071
Histology	-0.31	0.004	-0.518 to -0.1			
Differentiation	-0.05	0.694	-0.286 to 0.191	-0.01	0.959	-0.285 to 0.271
Smoking	0.425	0.004	0.143 to 0.707	0.387	0.01	-0.0962 to 0.677
pT classification	-0.02	0.763	-0.167 to 0.123	-0.15	0.095	-0.322 to 0.026
pN classification	0.022	0.727	-0.101 to 0.144	0.145	0.088	-0.022 to 0.313
Ki-67	2E-04	0.929	-0.004 to 0.004	-0.01	0.077	-0.011 to 6E-04
Cyclin E	0	0.652	-0.004 to 0.003	0.002	0.424	-0.003 to 0.006
GalNAcT3	-0.23	0.032	-0.483 to -0.02	-0.111	0.507	-0.423 to 0.212
GnT-V	-0.03	0.483	-0.111 to 0.053	0.007	0.889	-0.095 to 0.11
Survival time	2E-06	0.948	-7E-05 to 8E-05	4E-05	0.383	-6E-05 to 1E-04

In summary, we showed that AKR1B10 is overexpressed in most SCCs and in adenocarcinomas that developed in the lung of smokers. Considering its involvement in retinoic acid metabolic pathway, AKR1B10 could be not a mere surrogate marker but a molecule relevant in smoking-related NSCLCs. Elucidation of its roles in carcinogenesis will be required to evaluate AKR1B10 as a therapeutic target in addition to a potential marker of SCC for diagnosis as shown in this study.

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Global gene expression analysis of rat colon cancers induced by a food-borne carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine

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Colon cancers develop after accumulation of multiple genetic and epigenetic alterations in colon epithelial cells. To shed light on global changes in gene expression of colon cancers and to gain further insight into the molecular mechanisms underlying colon carcinogenesis, we have conducted a comprehensive microarray analysis of mRNA using a rat colon cancer model with the food-borne carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). Of 8749 genes or ESTs on a high density oligonucleotide microarray, 27 and 46 were over- and under-expressed, respectively, by ≥ 3 -fold in colon cancers in common in two rat strains with distinct susceptibility to PhIP carcinogenesis. For example, genes involved in inflammation and matrix proteases and a cell cycle regulator gene, *cyclin D2*, were highly expressed in colon cancers. In contrast, genes encoding structural proteins, muscle-related proteins, matrix-composing and mucin-like proteins were underexpressed. Interestingly, a subset of genes whose expression is characteristic of Paneth cells, i.e. the *defensins* and *matrilysin*, were highly overexpressed in colon cancers. The presence of *defensin 3* and *defensin 5* transcripts in cancer cells could also be confirmed by *in situ* mRNA hybridization. Furthermore, Alcian blue/periodic acid Schiff base (AB-PAS) staining and immunohistochemical analysis with an anti-lysozyme antibody demonstrated Paneth cells in the cancer tissues. AB-PAS-positive cells were also observed in high grade dysplastic aberrant crypt foci, which are considered to be preneoplastic lesions of the colon. Our results suggest that Paneth cell differentiation in colon epithelial cells could be an early morphological change in cryptic cells during colon carcinogenesis.

Introduction

The development of colon cancers comprises multiple steps requiring the accumulation of genetic and epigenetic alterations in colon epithelial cells, and these changes further affect

Abbreviations: AB-PAS, Alcian blue/periodic acid Schiff base; ACF, aberrant crypt foci; DIG, digoxigenin; APC, adenomatous polyposis coli; EST, expressed sequence tagged; H&E, hematoxylin and eosin; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine.

expression of a variety of downstream genes and may cause considerable changes in gene expression profiles in cancer cells as a consequence. Inactivation of the *adenomatous polyposis coli* (APC) gene, β -*catenin*, K-RAS, SMAD2, SMAD4, *p53* and mismatch repair genes by genetic alterations, for example, play key roles (1,2). Furthermore, alterations of gene expression profiles by perturbation of CpG island methylation in promoter regions and/or the histone acetylation/deacetylation status of chromatin also have a substantial impact on colon carcinogenesis (2).

Oral administration of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), one of the most abundant heterocyclic amines produced while cooking meat and fish (3,4), induces aberrant crypt foci (ACF) (5,6), putative preneoplastic lesions of the colon (7,8), in experimental animals within a short period and colon adenomas and adenocarcinomas after 1 or 2 years in rats, preferentially in males (9). A number of studies have revealed that PhIP-induced rat colon cancers resemble human neoplasms with regard to observed histological features and genetic alterations (10–14). There are several advantages with the use of animal cancer models to dissect the molecular basis of colon carcinogenesis. For example, inbred experimental animals share a common genetic background within the strain and, furthermore, carcinogenesis experiments using these animals can be carried out under well-controlled conditions. Genetic and/or epigenetic alterations in colon cancers induced in experimental animals are therefore expected to be more uniform compared with those in humans with diverse genetic backgrounds. Colon cancers induced by PhIP indeed demonstrate β -*catenin* accumulation in both cytoplasm and nucleus (13) and β -*catenin* mutations are observed at codons 32, 34, 36 or 38 in exon 2, the majority being G \rightarrow T transversions (12,13). In the *Apc* gene, 5'-GGGA-3' sites in exons 14 and 15 and a 5'-agGGGG-3' site at the junction of intron 10 and exon 11 are mutation hot-spots (10,13). Using a model system, we have recently revealed sequential progression from dysplastic ACF to colon cancer (14,15). Although the PhIP-induced rat colon cancer model has provided cancer researchers with a powerful tool for dissecting molecular events involved in the formation of colon cancers with relevance to human colon carcinogenesis, extensive studies aimed at the elucidation of early genetic events in colon cancer development have hitherto not been conducted.

In the present study we therefore performed a global gene expression analysis of rat colon cancers induced by PhIP using high density oligonucleotide microarrays (GeneChip; Affymetrix, Santa Clara, CA). To eliminate detection of strain-specific changes, but rather to detect specific gene expression profiles essential for colon cancer development, two rat strains, F344 and ACI, were subjected to analysis, the former being the more susceptible to PhIP-induced colon carcinogenesis. A considerable number of genes were found to be differentially expressed in colon cancers compared with normal counterpart epithelium, including examples characteristic of Paneth cells. Global

changes in gene expression profiles are also discussed in comparison with those reported in human colon cancers. Another focus is on the appearance of Paneth cells in ACF, especially in dysplastic ones, and its biological significance.

Materials and methods

Animals and diets

PhIP was purchased from the Nard Institute (Osaka, Japan) in the form of PhIP-HCl and added to AIN-93G basal diet (7% w/w soybean oil; Dyets, Bethlehem, PA) at a concentration of 400 p.p.m. A high fat diet (AIN-93G basal diet supplemented with 23% w/w hydrogenated vegetable oil) was also purchased from Dyets. Five-week-old male F344 and ACI strain rats were purchased from CLEA Japan (Tokyo, Japan) and housed 3 per cage in an air-conditioned animal room with a 12 h light/dark cycle. Prior to the experiment, all the animals were acclimatized to the housing environment and the AIN-93G basal diet for 1 week.

Experimental protocol and tissue samples

Starting at the age of 6 weeks, rats were fed a diet containing PhIP following an intermittent PhIP feeding protocol (13). At experimental week 60 all animals were killed and colons were removed. When colon cancers with polypoid growth were detected by the naked eye, cancerous parts were resected with a razor blade, bisected and one half was embedded in O.C.T. compound (Tissue-Tek; Sakura Finetechnical Co., Tokyo, Japan), frozen and stored at -80°C until use for frozen section preparation and RNA extraction. The remaining halves were fixed in neutral 10% formalin overnight at 4°C and embedded in paraffin blocks according to standard procedures. Normal counterparts were collected from the surrounding normal parts of the colon and separately embedded in O.C.T. compound and samples were snap-frozen in liquid nitrogen and stored at -80°C until use for RNA extraction. In separate experiments using the intermittent PhIP feeding protocol, ACF were assayed at experimental weeks 18 and 25, after fixation of tissue in formalin and embedding in paraffin blocks as described above.

High density oligonucleotide microarray analysis

Twelve colon cancer tissues, six each from F344 and ACI rats, and 12 normal counterparts were collected by digging them out of frozen O.C.T. blocks using 18 gauge needles. Total RNA was extracted from ~ 1 mg of tissue with TRIZOL reagent (Invitrogen, Carlsbad, CA). Two of six colon cancer tissues from F344 rats, however, did not provide sufficient amounts of good quality RNA. The remaining four samples from F344 and six from ACI rats were subjected to the following experiments. cRNA was synthesized, labeled with biotin and hybridized to high density oligonucleotide microarrays, Rat Genome U34A (RG U34A; Affymetrix), as described previously. The average hybridization intensity for each array was scaled to 1000 to reliably compare multiple arrays. Prior to statistical analysis, genes were filtered according to the following criteria. For genes overexpressed in cancers, for example, they should have 'present (P)' or 'marginal (M)' calls in at least half of the colon cancer samples of the respective rat strains. For genes underexpressed in cancers, in contrast, they should have P or M calls in half of the normal counterpart samples. To assess statistical differences in gene expression between colon cancers and normal tissues, average signal intensity and standard variation were calculated for each group and GeneSpring 4.3

(Silicon Genetics, Redwood City, CA) was employed for the Mann-Whitney *U*-test. The significant *P* value was set at 0.05. Then, genes which were differentially expressed between cancer and normal tissue at ≥ 3 -fold were selected and subjected to further analysis, including Venn diagrams, hierarchical clustering analysis, functional classification and comparison with expression profiles of human colon cancers. Permutation analysis was also carried out to assess the statistical significance of genes differentially expressed between the two rat strains.

Histological analysis

For hematoxylin and eosin (H&E) staining, paraffin sections were prepared at $3.5\ \mu\text{m}$ thickness following standard procedures. Histological evaluation of colonic lesions was performed as described previously (13). For Alcian blue (pH 2.5)/periodic acid Schiff base (AB-PAS) staining to evaluate the presence of Paneth cells, both frozen ($10\ \mu\text{m}$ thickness) and paraffin ($3.5\ \mu\text{m}$ thickness) sections were used. The staining was carried out according to conventional methods.

In situ mRNA hybridization for defensin genes

In situ mRNA hybridization was carried out as described previously (16,17) under contract by Genostaff (Tokyo, Japan) using frozen sections prepared at $10\ \mu\text{m}$ thickness. A 293 bp cDNA fragment of the rat neutrophil defensin 3 gene was amplified by PCR with primers 5'-CTCCCTGCATACGCCAAAG-3' (forward) and 5'-AACAGAGTCGGTAGATGCG-3' (reverse) and a 335 bp cDNA fragment of the defensin 5 gene with primers 5'-AACTGTCTCCTTCTGCC-3' (forward) and 5'-AACATCAGCATCGGTGGCC-3' (reverse). Amplified fragments were cloned into pCRII (Invitrogen) and digoxigenin (DIG)-labeled RNA probes were generated by an *in vitro* transcription method using DIG-labeling mix (Roche Molecular Biochemicals, Tokyo, Japan). Hybridized probes were detected by an IgG antibody against the DIG label and visualized with NBT/BCIP solution (Roche Molecular Biochemicals). Nuclear counterstaining was performed with Kermehrot Stain Sol (Muto Chemical, Tokyo, Japan).

Semi-quantitative RT-PCR

Extracted RNA was transcribed to cDNA using an oligo(dT)₁₂₋₁₈ primer and SuperScript™ II reverse transcriptase (Invitrogen) and the cDNAs produced were divided into aliquots in tubes and stored at -20°C until analyzed. Each aliquot of cDNA was subjected to semi-quantitative reverse transcription (RT)-PCR with the primer sequences listed in Table I. A set of semi-quantitative RT-PCR reactions for representative genes was carried out within 1 day to avoid the effects of degradation of cDNA templates. For reference, expression of the β -actin and glyceraldehyde 3-phosphate dehydrogenase (*G3PDH*) genes was also quantified for each sample. PCR amplification was carried out at 94°C for 30 s, 60°C for 30 s and 72°C for 1 min using Advantage Taq (Clontech, Palo Alto, CA) under the conditions recommended by the manufacturer. PCR cycles were set at 25 for β -actin and *G3PDH*, 35 for α -defensin NP4 and β -defensin 2 and 30 cycles for the other genes. PCR products were also analyzed by gel electrophoresis on a 2% agarose gel in $0.5\times$ TBE (89 mM Tris, 89 mM boric acid, 1.9 mM EDTA). The amounts of PCR products were quantified by analysis performed on a Macintosh iBook G3 computer using the public domain NIH Image program (developed at the US National Institutes of Health and available on the Internet by anonymous ftp from zippy.nimh.nih.gov. or on floppy disk from the National Technical Information Service, Springfield, VA, part no. PB95-500195GE1). PCR reactions for individual genes were

Table I. List of primers used for RT-PCR

Gene name	Forward primer	Reverse primer
Matrilysin	5'-TTCGCAAGGGGAGATCACG-3'	5'-AACAGAAGAGTGACCCAGAC-3'
Mash2	5'-TTACCCATGCTGTCTAGTGC-3'	5'-AGTCCTCCAGCAGTTCAGT-3'
Oct1A	5'-CCTTCATCATCCTGTGTCAC-3'	5'-ATGAAGGGGGTGAAGATCC-3'
Carbonic anhydrase IV	5'-GGTAAACGAGGGCTTCCAG-3'	5'-TGAGACCTGAACACCTGGC-3'
AA799832	5'-GCGATCATGCCTTGCTAAC-3'	5'-TTCCAGCGGCAGTGAAGG-3'
Defensin NP1 like	5'-TGCTGTTCAAGATTTACGCG-3'	5'-ACCTTGATAGCCOATGCAGC-3'
Defensin NP3	5'-CTCCCTGCATACGCCAAAG-3'	5'-AACAGAGTCGGTAGATGCG-3'
Defensin $\alpha 5$	5'-AACTGTCTCCTTCTGCC-3'	5'-AACATCAGCATCGGTGGCC-3'
Defensin NP4	5'-GACACTCACTCTGCTCATCA-3'	5'-ATGACAAAATGGCTTCTTCTC-3'
Defensin $\beta 1$	5'-CTTGGACGCAGAACAGATCA-3'	5'-AAACCACTGTCAACTCTGC-3'
β -Actin	5'-GACTTCGAGCAAGAGATGCC-3'	5'-AGGAAGGAAGGCTGGAAGAG-3'
G3PDH	5'-TCATGACCACAGTCCATGCC-3'	5'-CTCAGTGTAGCCAGGATGC-3'

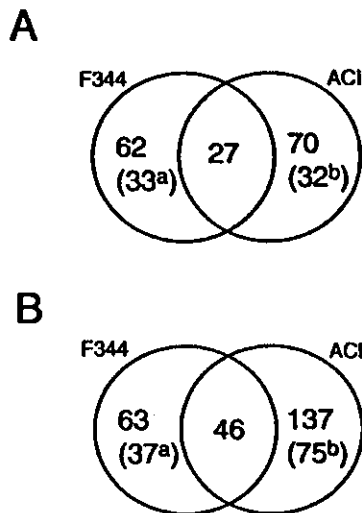


Fig. 1. Numbers of genes over- or underexpressed in colon cancers. Numbers of genes (A) overexpressed or (B) underexpressed in colon cancers of F344 or ACI rats are indicated in Venn diagrams. These genes showed significant differences ($P < 0.05$, Mann-Whitney U -test) between colon cancer tissues and normal colon tissues of ≥ 3 -fold. The numbers in parentheses indicate the numbers of genes that also showed ≥ 2 -fold differences in ACI (a) and F344 (b), respectively.

repeated twice, all of which gave similar results, and representative data are shown.

Results

Gene expression profiles in colon cancers of two rat strains

Of 8749 genes or ESTs on the RG U34A array, 89 and 97 were overexpressed ≥ 3 -fold in colon cancers of F344 and ACI rats, respectively, and 109 and 183 were underexpressed by ≤ 3 -fold, as shown in the Venn diagrams (Figure 1A and B; GeneChip data are available at <http://www.ncc.go.jp/jp/nccri/divisions/02bioc/02bioc.html>). As illustrated in Figure 1(A), 27 were overexpressed ≥ 3 -fold in common in both F344 and ACI lesions. Although 62 genes were shown to be preferentially overexpressed in F344-derived colon cancers, 33 were also overexpressed ≥ 2 -fold, in both strains (Figure 1A). Similarly, 32 of 70 genes overexpressed ≥ 3 -fold in ACI rats were also overexpressed in the F344 strain by ≥ 2 -fold. Collectively, of 159 genes which showed ≥ 3 -fold expression in cancer tissues in either of the rat strains (Figure 1A), 92 (57.8%) showed ≥ 2 -fold expression in colon cancers in both strains. Similarly, 158 of 246 (64.2%) genes demonstrated ≤ 2 -fold expression in colon cancers in both strains (Figure 1B). We have recently reported that a considerable number of genes are differentially expressed in normal parts of colon epithelial cells between F344 and ACI rats after PhIP treatment (18) and the repertoire of differentially expressed genes may partly account for the different susceptibilities of the two rat strains. In contrast, differences in cancer tissues were relatively small compared with those in normal tissues. Permutation analysis was unable to detect apparent differences in the number of differentially expressed genes between cancer tissues of F344 and ACI rats, although significant differences were present in normal colon epithelium (data not shown). Hierarchical clustering analysis using the entire list of over- and underexpressed

genes in cancers could not elucidate any cluster(s) of genes specific for either strain (data not shown).

Genes overexpressed in colon cancers

As described above, 27 genes were overexpressed ≥ 3 -fold in common in both F344 and ACI rats (Figure 1A). Despite our efforts to elucidate meaningful signal pathways in those 27 genes using the bioinformatics software package GenMapp (<http://www.genmapp.org/>), none could be identified. This could be due, at least in part, to insufficient coverage of genes in signal pathway databases for rats. Manual classification, however, was able to elucidate some interesting tendencies. Genes involved in inflammation, such as those encoding interleukin 1 β , small inducible cytokine subfamily A20 precursor, and proteases, such as *matrilysin (Mmp7)* and *macrophage metalloelastase (Mme)* were highly overexpressed in cancer tissues (Table II). A cell cycle regulator, *cyclin D2*, and the cancer-related gene *retrotransposon virus-like 30s sequence (VL30)*, which is known to be overexpressed in rodent hepatocellular tumors and lymphomas (19,20), were also highly expressed. Interestingly, a subset of genes encoding defensin and defensin-like proteins, which belong to the small cationic antimicrobial cytotoxic peptides (21–23), were overexpressed ≥ 10 -fold. Differential expression of some of the representative genes was confirmed by semi-quantitative RT-PCR (Figure 2A).

Genes underexpressed in colon cancers

Forty-six genes were underexpressed ≤ 3 -fold in common in colon cancers of both the F344 and ACI strains (Figure 1B). Genes encoding metabolic enzymes, such as alanine aminotransferase, minoxidil sulfotransferase and carbonic anhydrase IV, and signal transduction molecules were among the list (Table III). A considerable number of transcripts related to the structural proteins, i.e. skeletal and smooth muscle-related proteins, matrix-composing proteins and mucin-like proteins, were underexpressed in colon cancers. Down-regulation of mucin genes in colon cancers may reflect the drastic decrease in or complete loss of goblet cells in cancer tissues. Representative results of RT-PCR analyses are depicted in Figure 2A.

Expression of oncogenes and tumor suppressor genes

Expression of tumor-related genes, including those considered to be involved in colon carcinogenesis, was also evaluated utilizing the Chip data. Signals for the *Apc*, *bcl2*, *c-jun*, *erbB3*, *VHL* and *WT1* genes were below detectable levels. The *p53* and *c-fos* genes were expressed at comparable levels in both colon cancers and normal parts of colon tissues (data not shown). Only the *c-myc* gene was expressed at a significantly higher level in cancer tissues than in normal counterparts in both rat strains, with 4.0- and 2.5-fold differences in F344 and ACI rats, respectively.

Comparison of gene expression profiles with human colon cancers

Some of the genes in the list have already been reported to be either over- or underexpressed in human colon cancers (24,25). Average fold changes between colon cancers and normal counterparts were calculated using combined data from both the F344 and ACI strains and genes with ≥ 5 -fold differences are listed in Table IV and compared with human cases, referring to the literature (24–31). The *defensin $\alpha 5$* and *defensin $\alpha 6$* genes were previously reported

Table II. Genes overexpressed in tumors of both F344 and ACI rats^a

Gene name	Average signal intensity				Fold change (T/N) ^b	
	F344		ACI		F344	ACI
	Normal	Tumor	Normal	Tumor		
HLA and immune function genes						
Anti-acetylcholine receptor antibody gene, rearranged Ig Gamma-2a chain, VDJC region	246.73	3738.53	200.00	3929.67	15.15	19.65
Cytokines, inflammatory mediators, antimicrobial						
(EST) A1639089/similar to mouse defensin NP1 (α 1)	1765.02	35820.01	200.00	55038.35	20.29	275.19
Defensin NP3 (α 3) gene	898.73	14754.13	919.32	12107.13	16.42	13.17
Interleukin 1- β mRNA	200.00	1225.78	200.00	1658.88	6.13	8.29
Small inducible cytokine subfamily A20	573.80	2328.28	582.04	3176.26	4.06	5.46
Mob-1	214.46	758.60	200.00	979.33	3.54	4.90
Detoxification enzymes						
Glutathione S-transferase M5 (Gst-M5)	334.82	2013.20	259.33	1366.90	6.01	5.27
Protease and protease inhibitors						
Matrilysin (Mmp-7) mRNA	200.00	5823.69	200.00	15258.13	29.12	76.29
Macrophage metalloelastase (Mme)	200.00	2047.38	200.00	1990.87	10.24	9.95
Maspin	677.18	1954.74	637.72	2957.50	2.89	4.64
Ion transporters, carrier proteins						
Cation transporter Oct1A	1180.79	6642.65	756.38	6553.23	5.63	8.66
Intracellular calcium-binding protein Mrp14	200.00	1004.26	200.00	2011.03	5.02	10.06
Signal transduction molecules, transcription factor						
Mash-2 mRNA expressed in neuronal precursor cells	200.00	2867.48	303.83	3381.59	14.34	11.13
Platelet phospholipase A2	6078.53	54470.52	7630.13	99549.62	8.96	13.05
Receptor-linked protein tyrosine phosphatase	281.53	2171.51	200.00	2747.78	7.71	13.74
Inhibitor of DNA binding 3 (Idb3)	1585.27	7556.29	1819.98	9419.78	4.77	5.18
Hypertension-regulated vascular factor-1 (Ruk)	1956.67	7039.99	961.72	4728.72	3.60	4.92
Cell cycle regulators						
Cyclin D2	200.00	1281.43	200.00	1218.13	6.41	6.09
Similar to cyclin D2 (Vin1)	5132.35	19215.31	3399.26	18358.46	3.74	5.40
Cancer-related genes (function unknown)						
VL30 element	1055.79	10942.00	1234.52	6470.70	10.36	5.24
c-Ha-ras protooncogene mechanism sequence	4157.40	32175.09	2926.38	26356.43	7.74	9.01
Structural proteins						
Type I keratin (Mhr a-1)	200.00	3584.60	200.00	1440.44	17.92	7.20
β -Tubulin T β 15	2176.01	8522.65	3252.25	11450.90	3.92	3.52
Serum protein						
α 2-Macroglobulin	903.14	5880.35	1023.80	6303.21	6.51	6.16
Function unknown						
(EST) AA859937	572.42	5572.50	251.35	5924.12	9.73	23.57
(EST) AA799396	955.18	4061.69	1094.52	5774.63	4.25	5.28

^aGenes with ≥ 3 -fold difference compared with those in normal counterpart tissues are listed.

^bFold changes were calculated by dividing the signal intensity of colon cancer tissue by that of normal colon tissue.

to be up-regulated in human colon cancers [SAGE data (25)]. In the present study, *defensin NP3* ($\alpha 3$) and *defensin NP1* ($\alpha 1$)-like molecule were revealed to be overexpressed, although they have not been reported to be overexpressed in human cases. The matrix proteases *Mmp-7* and *Mme* [DNA chip data (24)] and *Mash2*, *Mrp14* and *cyclin D2* [SAGE data (25)] were also reported to be highly expressed in human colon cancers. In the case of underexpressed genes, many of them were also reported to be down-regulated in human cancers, such as *mucin*, *guanylin*, *carbonic anhydrase IV* and several muscle- and structure-related genes (24,32).

Expression of defensin family genes in rat colon cancers

Defensin genes are composed of mainly two families, α and β , categorized by sequence similarities (23,33). Genes in the former group are expressed mainly in neutrophils and some in the intestine, while the latter are ubiquitously expressed. *defensin NP1* ($\alpha 1$)-like molecule and *defensin NP3* ($\alpha 3$) was

found to be highly expressed in colon cancers of both strains. *In situ* hybridization analysis revealed mRNAs of *defensin NP3* ($\alpha 3$) and *defensin $\alpha 5$* to be expressed exclusively in epithelial cells of colon cancers and not expressed in matrix cells (Figure 3A). Normal epithelium did not show any positive signals. Overexpression of these genes in colon cancer tissues was confirmed by RT-PCR (Figure 2B). *defensin $\alpha 5$* , which is an intestinal-type defensin, but is not on the Gene-Chip, was also expressed exclusively in colon cancers. No expression was observed for *defensin NP4* ($\alpha 4$), *defensin $\beta 1$* (Figure 2B) or *defensin $\beta 2$* (data not shown) in either cancers or normal counterpart tissues.

Presence of Paneth cells in cancers and preneoplastic lesions of the colon

Since intestinal-type defensins are known to be produced in Paneth cells (23,34), we examined the expression of other marker proteins specific to the Paneth cell lineage. H&E and

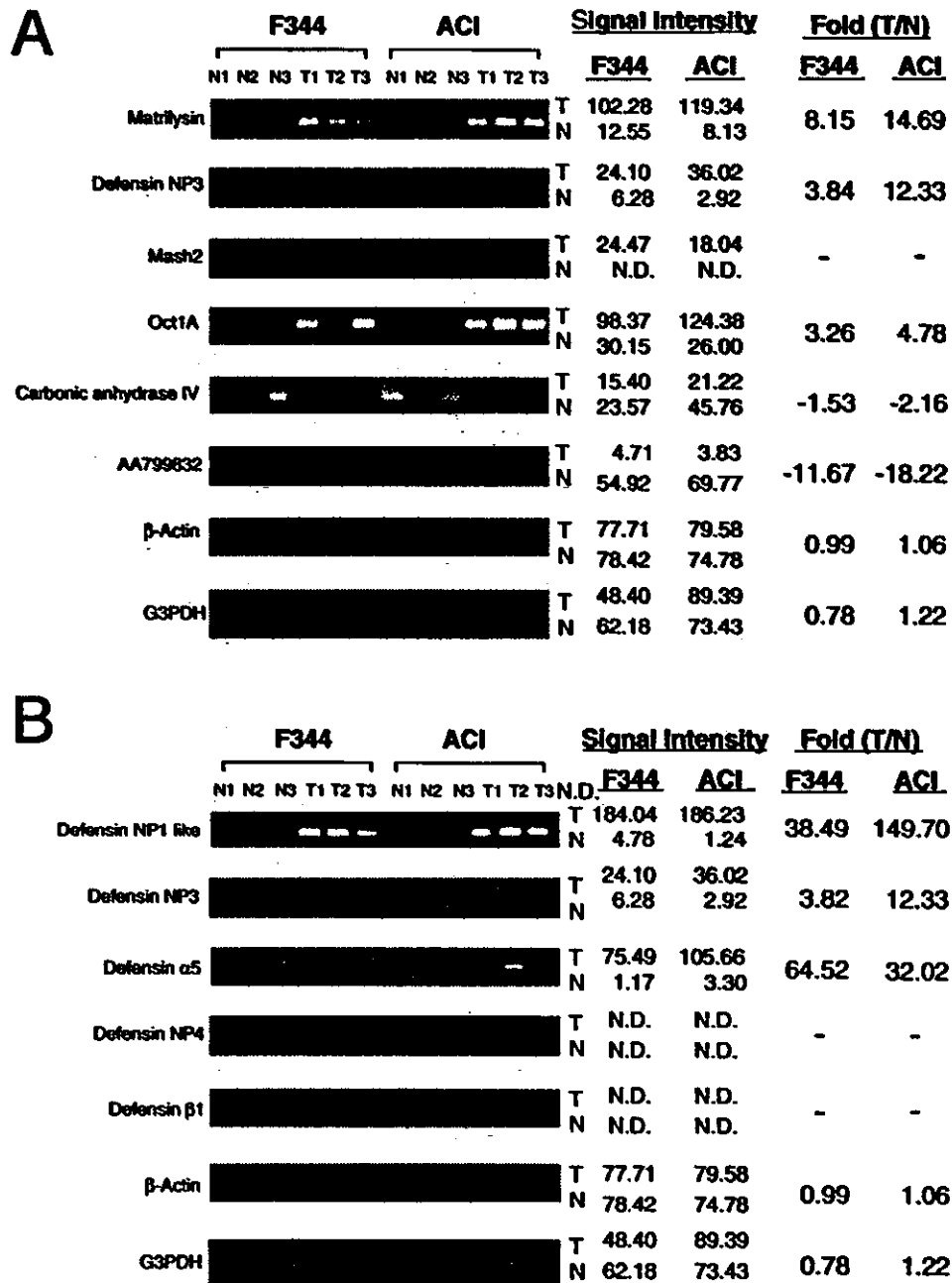


Fig. 2. Semi-quantitative RT-PCR analysis. (A) Among genes over- or underexpressed in colon cancers, expression levels of representative examples were confirmed by semi-quantitative RT-PCR analysis. Each lane indicates the gene expression level of either tumor or normal counterpart tissue from individual animals. The upper four panels illustrate higher expression of *matrilysin*, *defensin NP3*, *Mash2* and *Oct1A*, respectively, in cancer tissues (T₁₋₃) compared with normal epithelium (N₁₋₃). In contrast, *carbonic anhydrase IV* and *phosphoglucomutase 1* were underexpressed in cancers. Expression of *β-actin* and *G3PDH* was analyzed as an internal control. The amounts of PCR products were quantified by NIH image software. The signal values were calculated by subtracting blank values, which are average signal intensities of three different points outside the signal bands. Fold differences were calculated by dividing the average of the value of colon cancer tissues (T) by that of normal colon tissues (N). N.D. indicates a sample with no PCR product visible to the naked eye. (B) Expression levels of several *defensin* genes were analyzed by RT-PCR. *Defensin NP1-like molecule*, *defensin NP3*, *defensin 5* and *defensin NP4* are *α-defensin* family genes and *defensin β1* belongs to the *β-defensin* family gene. Quantification of the amounts of PCR products was carried out as above.

AB-PAS staining revealed the presence of Paneth granules in colon cancer cells and lysozyme expression was also observed in cells with Paneth granules (Figure 3B). Furthermore, Paneth cells were observed in adenomas and, to our surprise, even in preneoplastic lesions. When examined by H&E and AB-PAS

staining, three of eight colon cancers and two of three high grade dysplastic ACF observed at 18 or 25 weeks were demonstrated to contain Paneth cells within the lesion (Figure 4A). None of the non-dysplastic ACF demonstrated Paneth cell differentiation (Figure 4B).

Table III. Genes underexpressed in tumors of both F344 and ACI rats^a

Gene name	Average signal intensity				Fold change (T/N) ^b	
	F344		ACI		F344	ACI
	Normal	Tumor	Normal	Tumor		
Stress proteins and antioxidant						
Heat shock protein 27	8538.00	1967.31	11801.28	2648.38	4.34	4.46
Hsp70.2 mRNA for heat shock protein 70	766.00	200.00	3572.83	210.58	3.83	16.97
Metabolic enzymes						
Alanine aminotransferase mRNA	2078.57	200.00	1557.94	200.00	10.39	7.79
Minoxidil sulfotransferase	2170.13	208.88	1748.53	357.00	10.39	4.90
Carbonic anhydrase IV	2467.84	302.40	2651.28	220.07	8.16	12.05
Inducible carbonyl reductase	1676.98	234.19	1690.85	424.75	7.16	3.98
Skeletal muscle creatine kinase composite	1127.20	237.98	903.73	249.44	4.74	3.62
Mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase	8830.96	2414.46	9434.23	939.58	3.66	10.04
Monoamine oxidase B	680.05	200.00	918.93	200.00	3.40	4.59
Lipoprotein lipase	2259.29	684.09	2316.56	225.35	3.30	10.28
Protease						
Endopeptidase-24.18 α subunit	3327.41	773.59	5676.14	570.71	4.30	9.95
Ion transporters, carrier proteins						
SC1 protein	45395.45	3784.08	24395.65	1981.58	12.00	12.31
Dihydropyridine-sensitive L-type Ca ²⁺ channel α -2 subunit	1420.65	229.11	3067.40	200.00	6.20	15.34
Guanylin	26005.42	5019.71	34049.06	6235.18	5.18	5.46
Growth factors and hormones						
Insulin growth factor-binding protein	2446.05	240.24	3255.07	200.00	10.18	16.28
Ebnerin	31568.63	3536.31	5773.52	1388.94	8.93	4.16
Signal transduction molecules, transcription factors						
Neuron-specific protein Pep-19	3125.96	200.00	2721.96	200.00	15.63	13.61
Phospholipase C- β 1b	1714.75	244.15	2137.83	323.40	7.02	6.61
D-binding protein	1368.84	200.00	1038.96	200.00	6.84	5.19
Guanylate cyclase 1, soluble, α 3	2375.39	384.13	1670.49	519.78	6.18	3.21
Gap-43 gene	1666.24	271.56	982.00	293.22	6.14	3.35
Sscks 322	6158.29	1093.83	3320.23	522.72	5.63	6.35
N-myc downstream regulated 2	2273.34	486.21	3961.73	710.56	4.68	5.58
Tcl10-like rho GTPase	2287.18	582.64	4295.60	511.18	3.93	8.40
BTE-binding protein	2136.52	625.70	3136.92	668.54	3.41	4.69
Structural proteins						
H36- α 7 integrin α chain	11056.70	501.93	4844.89	392.89	22.03	12.33
α -Crystallin B chain	4016.12	200.00	3133.37	383.01	20.08	8.18
α b-Crystallin-related protein	4322.27	375.25	3731.25	200.00	11.52	18.66
Mucin-like protein	85858.60	26168.84	85879.95	15703.47	3.28	5.47
Muscle-related proteins						
Skeletal muscle β -tropomyosin and fibroblast tropomyosin 1	15036.62	578.13	8208.40	331.54	26.01	24.76
Myosin regulatory light chain isoform C	32928.30	1592.13	21440.35	576.02	20.68	37.22
γ -Enteric smooth muscle actin isoform	107504.58	5932.44	64435.47	1234.91	18.12	52.18
Calponin	22307.16	1283.39	14767.63	349.48	17.38	42.26
SM22 mRNA	25962.76	1742.56	9714.69	200.00	14.90	48.57
Alternatively spliced smooth muscle myosin heavy chain	21642.04	1629.01	13526.24	591.39	13.29	22.87
Vascular α -actin	28016.47	4213.15	28042.35	2126.16	6.65	13.19
Function unknown						
(EST) AA799832/similar to mouse phosphoglucomutase 5	17333.24	629.26	16572.28	200.00	27.55	82.86
(EST) AA799773/similar to mouse C3H filamin	4300.66	387.96	5571.79	558.87	11.09	9.97
(EST) AA800735/similar to archvillin	3155.42	366.63	3353.20	360.25	8.61	9.31
(EST) AA799580	2256.37	338.94	1619.85	420.47	6.66	3.85
(EST) AA799734	1290.58	200.00	950.38	200.00	6.45	4.75
(EST) AA892888	5611.57	949.04	3961.43	200.00	5.91	19.81
zymogen granule protein (Zg-16p)	14971.90	2743.63	25128.90	1816.38	5.46	13.83
(EST) AA893743/similar to human hox B2	1017.45	200.00	952.02	200.00	5.09	4.76
(EST) AI639501/similar to human brain cell membrane protein 1	4324.68	871.69	4080.30	1027.55	4.96	3.97

^aGene with ≥ 3 -fold difference compared with those in normal counterpart tissues are listed.^bFold changes were calculated by dividing the signal intensity of normal colon tissues by that of colon cancer tissues.

Discussion

With the present comprehensive gene expression analysis, conducted using PHIP-induced rat colon cancers in two rat strains, PHIP-induced colon cancers were found to possess

some common gene expression profiles in both F344 and ACI rats, despite the significant differences in gene expression between their normal colon epithelium (18). Furthermore, a subset of genes known to be overexpressed in

Table IV. Comparison of overexpressed and underexpressed genes in tumors of rat and human

Gene name	Rat Fold	Human		
		DNA chip ^a	SAGE ^b	Other data
Overexpressed				
Defensin NP1 (α 1)-like protein	53.30	Unchanged		
Matrilysin (Mmp-7) mRNA (EST) AA859937	41.89 14.37	8.00 c		Overexpressed (26,27)
Defensin NP3 (α 3) gene	13.47	Unchanged		
Platelet phospholipase A2	12.02	Unchanged		Overexpressed (28)
Type I keratin (Mhr a-1)	9.86	c		
Anti-acetylcholine receptor antibody gene	9.63	c		
Mash-2 mRNA expressed in neuronal precursor cells	9.62	c	9/0	
c-Ha-ras protooncogene mechanism sequence	8.90	c		
Intracellular calcium-binding protein Mrp14	8.18	Unchanged	11/0	
Receptor-linked protein tyrosine phosphatase	8.06	c		
Macrophage metalloelastase (Mme)	7.45	5.10		
Cation transporter Oct1A	7.43	c		
VL30 element	6.75	c		
Interleukin 1- β mRNA	6.05	Unchanged		
Cyclin D2	5.60	Unchanged	8/0	Overexpressed (29)
α 2-Macroglobulin	5.55	c		Overexpressed (30)
Under-expressed				
(EST)AA799832	40.36	2.7		
H36- α 7 integrin α chain	15.58	2.8		
Neuron-specific protein Pep-19 mRNA, complete cds	13.61	2.1		
Sc1 protein	12.18	4.5		Underexpressed (31)
Zg-16p	11.35	c		
α b-crystallin-related protein	11.34	c		
Dihydropyridine-sensitive L-type calcium channel	10.42	Unchanged		
α -Crystallin B chain	10.19	4.3		
(EST)AF119148/Musmus C3H filamin	9.56	5.8		
Alanine aminotransferase mRNA	9.35	c		
Insulin growth factor-binding protein	9.28	1.6		
(EST)AA892888	7.77	c		
Carbonic anhydrase IV	7.68	2		
(EST) AA800735/similar to mouse Supervillin	7.16	c		
Guanylin	6.04	20	55/0	
Phospholipase C- β 1b	5.78	c		
Ssecks 322	5.68	c		
D-binding protein	5.05	c	8/0	

The values indicate tag counts of normal tissues/tumor tissues.

^aData from Notterman *et al.* (24). Values indicate fold difference (normal/tumor).

^bData from Buckhaults *et al.* (25) and on-line SAGE database to which they submitted their results.

^cThere is no information in Notterman *et al.* (24) about these genes.

human colon cancers was highly expressed in colon cancers of both rat strains. In addition, there are substantial similarities in a list of underexpressed genes between human (24) and rat tumors (present study). The histological features of the rat cancers also showed a high similarity with human cases, as described previously (10,11,13,14). Taking all the data together, the PhIP-induced colon carcinogenesis model in rats thus appears an appropriate and relevant system for investigation of human colon carcinogenesis.

High expression of *cyclin D2* and *c-myc* may result from activation of the Wnt/ β -catenin signaling pathway as a consequence of β -catenin accumulation, this being a common feature in rat and human tumors (2,13,35). An mRNA species for a mucin-like protein, homologous to mouse mucin 2, was here shown to be underexpressed in cancer tissues. Mucins are known to be abnormally expressed in neoplastic lesions of the colon of humans (36). Moreover, since *mucin 2*^{-/-} mice demonstrated reduced numbers of goblet cells and spontaneously developed adenomas in the small intestine (37), down-regulation of the mucin-like protein mRNA may play an important role in PhIP colon carcinogenesis in rats.

Some of the genes found in the present study, including those encoding helix-loop-helix protein MASH2, organic cation transporter (OCT1A4), calcium-binding protein MRP14 and regulator of ubiquitous kinase (RUK), have not been reported so far to be differentially expressed in human colon cancers. Some of them are intriguing with regard to their biological functions, although there are few reports suggesting their involvement in human colon carcinogenesis. Considering the size (small), the non-invasive nature of rat lesions and the rare occurrence of *p53* or *K-ras* mutations in PhIP-induced colon cancers (38,39), differential expression of these genes in cancer tissues may suggest that their alteration occurs at an early stage of human colon carcinogenesis. Alternatively, of course, this could simply be a rodent-specific phenomenon. Further analysis is warranted in the future to clarify this point.

Snyderwine *et al.* recently reported gene expression profiles in PhIP-induced mammary gland tumors (40). Expression of a few genes overlapped between mammary gland (40) and colon tumors (present study) induced by PhIP, except for the *tubulin β 15* gene. In PhIP-induced rat mammary cancer, deregulation of cyclin D1/Cdk4 and phospho-Rb was postulated to play a

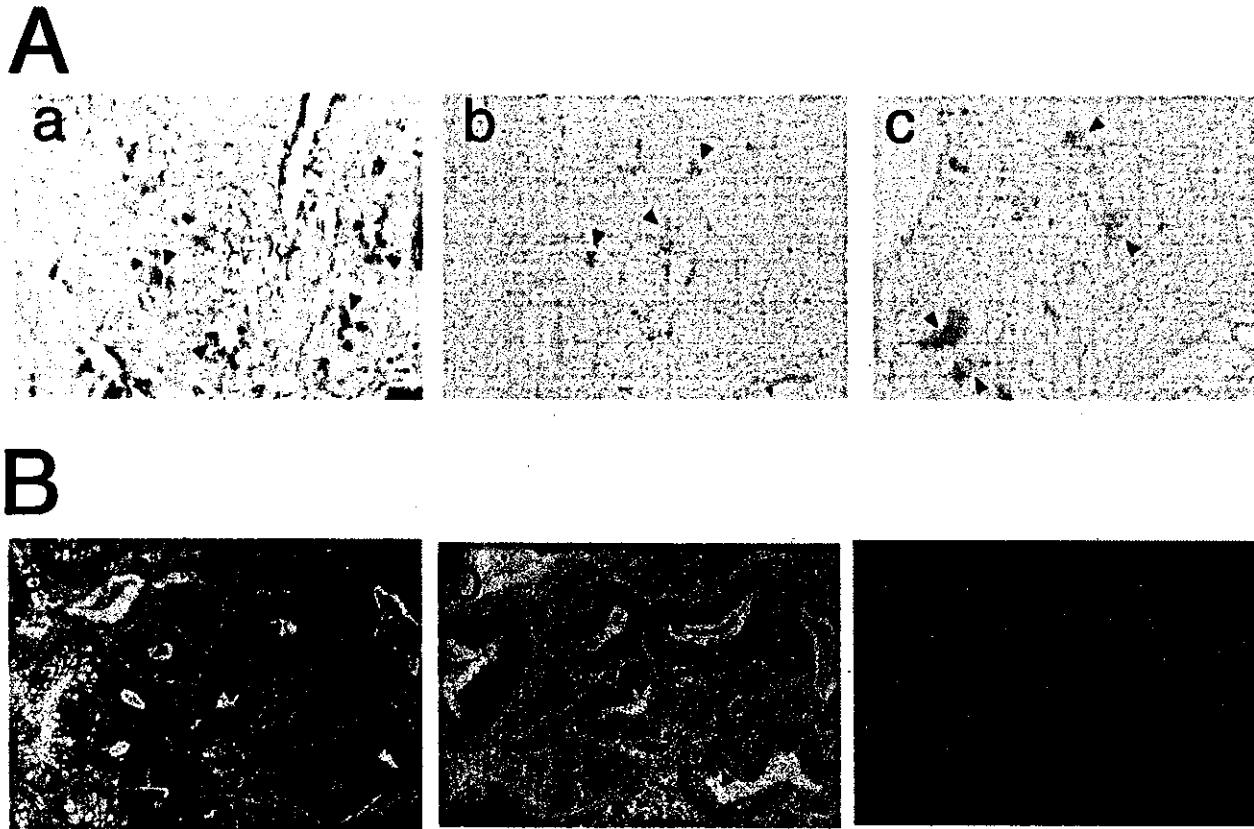


Fig. 3. Histological analysis of colon cancer tissues. (A) Frozen tissue sections were subjected to AB-PAS staining (a) and *in situ* hybridization with a *defensin NP3* (b) or *defensin 5* probe (c). Arrowheads in (b) and (c) indicate cells with *defensin NP3* and *defensin 5* transcripts, respectively. (B) Serial sections of paraffin embedded colon cancer tissues were subjected to H&E staining (a), AB-PAS staining (b) and immunostaining with an anti-lysozyme IgG antibody (c). As arrowheads indicate, Paneth cells can be recognized by the presence of typical pink granules (Paneth granules) by H&E staining. Paneth granules are also clearly visualized by AB-PAS staining (b) and by immunostaining for lysozyme protein (c).

central role (41). The results indicate that the molecular basis underlying PhIP carcinogenesis could differ from organ to organ, although cell proliferation may be accelerated by PhIP in both cases. In fact, β -catenin mutations are frequently observed in rat colon cancers (11–13), but only rarely in mammary tumors (42). Moreover, the mutation spectra found in PhIP-induced tumors also differ with the organ (43).

It is of great interest to note that Paneth cells were observed in colon cancers and even in much earlier lesions, dysplastic ACF. Paneth cells exist mainly in the small intestine and sometimes in colonic tumors, but are rarely found in normal colon epithelium (44,45). Yamada *et al.* have recently reported the presence of Paneth cells in crypts which accumulated β -catenin (BCAC) induced by an alkylating agent, azoxymethane (46,47). Based on their observations and a previous report by Wilson *et al.* (48), Yamada and Mori (49) suggested a dysdifferentiating potential of BCAC and that Paneth cell differentiation could promote intestinal carcinogenesis. Taking the results together, the appearance of Paneth cells in colon cancers does not appear to be carcinogen-specific, but could be a common phenomenon in the development of colon cancers. Although the molecular mechanisms underlying the induction of Paneth cells in colon cancers remain to be clarified, activation of the Wnt/Apc/ β -catenin signaling pathway could be one

causative event. Inhibition of β -catenin/TCF signaling in colon cancer cell lines indeed results in G₁ arrest or induction of markers which are characteristic of differentiated colon epithelial cells, as described previously (50). Mice deficient for the TCF4 transcription factor completely lack proliferating cells in the fetal small intestinal epithelium (51). The Wnt/APC/ β -catenin signaling pathway thus could be essential for maintenance of the differentiated or undifferentiated status of intestinal epithelial cells. Activation of the Wnt/APC/ β -catenin pathway may therefore affect the differentiation process and induce dysdifferentiation of colon epithelial cells as a consequence. Activation of this pathway is commonly observed in both PhIP- (10–15) and azoxymethane-induced (35,52,53) colon cancers and also in preneoplastic lesions (15). Gene expression analysis of teratomas, derived from embryonic stem cells with null APC, showed up-regulation of *defensin* α genes compared with teratomas derived from embryonic stem cells with wild-type APC (54). Paneth cells were also observed in APC-deficient teratoma tissues (54). Again, it is highly plausible that activation of the Wnt/APC/ β -catenin signaling pathway is a genetic causation of Paneth cell differentiation in colon cancer tissues. Although the biological consequences of Paneth cell differentiation (or metaplasia) for colon carcinogenesis remain to be clarified, it is

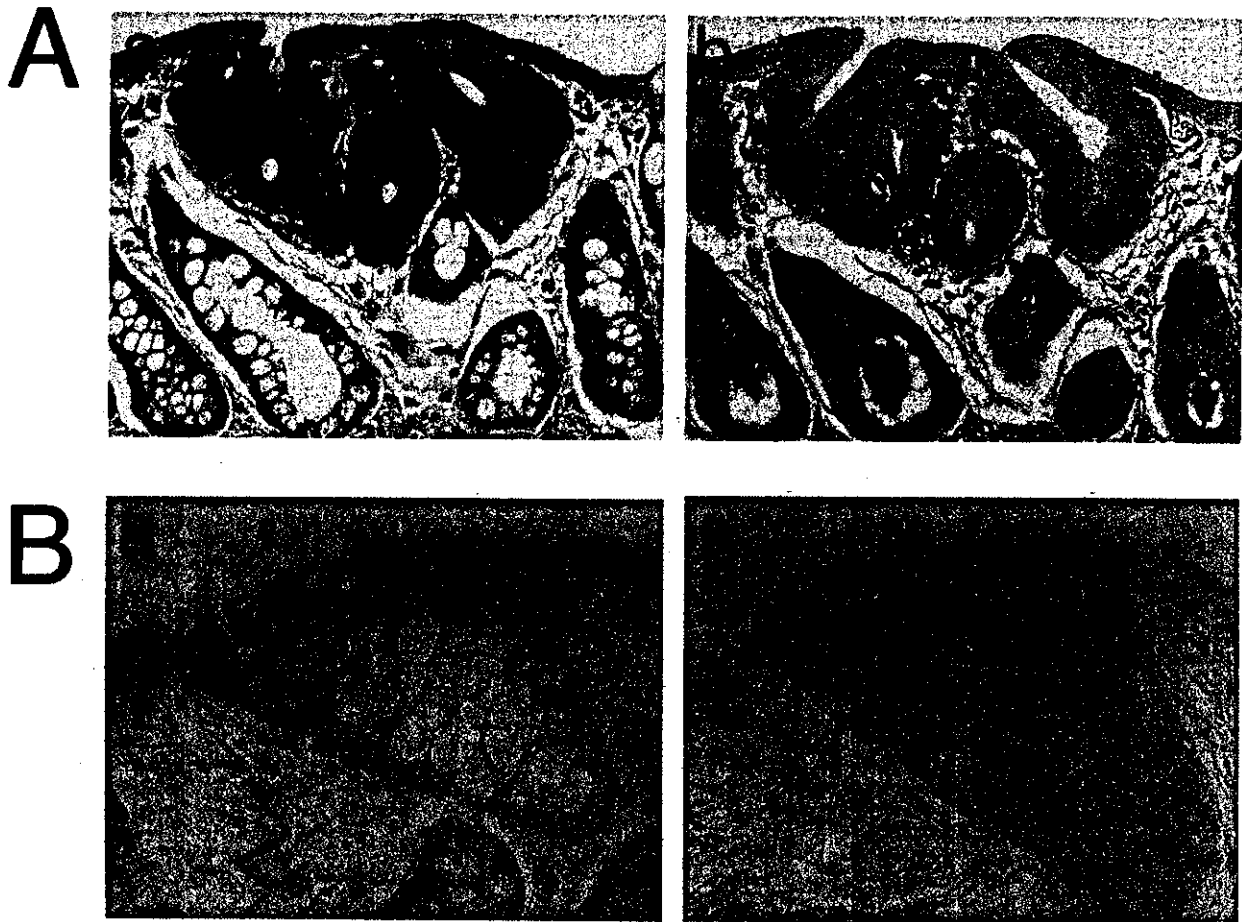


Fig. 4. Histological analysis of ACF. Serial sections of paraffin embedded high grade dysplastic (A) and non-dysplastic ACF (B) observed at experimental week 18. AB-PAS-positive Paneth granules are clearly evident in the high grade dysplastic ACF, but not in the non-dysplastic lesion. Another high grade dysplastic ACF collected at week 25 also gave similar results (data not shown). (a) H&E staining; (b) AB-PAS staining.

possible that the appearance of Paneth cells reflects aberrant differentiation of colonic stem cells. Whatever the case, defensin could be utilized as a potential serological marker for early detection of colon cancers because of its nature as a secreted molecule.

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