MOLECULAR CARCINOGENESIS 39:10–14 (2004)

Expression of the *p16^{INK4a}* Gene and Methylation Pattern of CpG Sites in the Promoter Region in Rat Tumor Cell Lines

Kanya Honoki,[1]* Toshifumi Tsujiuchi,[2] Toshio Mori,[3] Kazuhiro Yoshitani,[1] Masahiro Tsutsumi,[2] Yoshinori Takakura,[1] and Yoshio Mii[4]

[1]*Department of Orthopedic Surgery, Nara Medical University, Kashihara, Nara, Japan*
[2]*Department of Oncological Pathology, Nara Medical University, Kashihara, Nara, Japan*
[3]*Department of Radioisotope Research Center, Nara Medical University, Kashihara, Nara, Japan*
[4]*Department of Surgical Center, Nara Medical University, Kashihara, Nara, Japan*

Loss of p16^{INK4a} protein expression has frequently been related to DNA methylation in association with gene silencing. Although the methylation status of exon1α for p16^{INK4a} involvement in various cancers has been extensively analyzed, it has been pointed out that some inconsistencies existed in its relationship to gene silencing of *p16^{INK4a}*. In this study, we focused on the expression and methylation status in the regions of nt −478 to −201, containing a putative TATA box (nt −401 to −396), and nt −233 to 26, both in a recently cloned 5′ upstream region of rat *p16^{INK4a}*. We showed that rat lung adenocarcinoma RLCNR did not express the *p16^{INK4a}* gene, whereas rat osteosarcoma COS1NR and malignant fibrous histiocytoma MFH1NR both expressed it at levels similar to normal fibroblasts, even though the region of nt −233 to 26 was hypermethylated in COS1NR rather than RLCNR. In contrast, the CpG islands near the putative TATA box region were consistently methylated in RLCNR, but not in COS1NR and MFH1NR, as well as in normal fibroblasts. Treatment with 5-aza 2′-deoxycytidine induced expression of *p16^{INK4a}* gene in RLCNR after 48 h, but no changes were observed in COS1NR and MFH1NR. The results indicated that methylation of CpG islands near a TATA box region played a critical role for gene silencing of the rat *p16^{INK4a}* gene, rather than that of other regions.

Key words: p16^{INK4a}; methylation; gene silencing; TATA box; rat

INTRODUCTION

Cyclin-dependent kinases (cdks) comprise a family of enzymes that are core components of the cell-cycle machinery associated with cyclins [1]. Two families of cdk inhibitors, Cip/Kip and INK4 proteins, are known to regulate the cell-cycle progression negatively [2,3]. p16^{INK4a}, an INK4 family of cdk inhibitors, specifically inhibits cdk4 and cdk6 and is often inactivated in various cancer cells by deletion or mutation, or by the hypermethylation of CpG islands in its promoter region associated with gene silencing. The inactivation of the *p16^{INK4a}* gene provides a selective growth advantage in various cancer cells including lung tumors [4,5] and various types of sarcomas [6,7]. Among the various mechanisms of inactivation of the *p16^{INK4a}* gene, methylation-induced gene silencing has been extensively reported in almost all human cancer cells [8].

Gene exons are frequently hypermethylated at isolated CpGs (outside of CpG islands) and methylation of exon1α has been used for analysis of p16^{INK4a} involvement in rat lung [5], liver [9], and renal cancers [10]. However, recent studies have demonstrated that these methylated exons are not related to gene silencing [11,12] and it has been proposed that hypermethylation-mediated gene silencing is limited to CpG islands in gene promoter regions [13]. Several studies in human cases also indicate that methylation of a small number of CpG sites in the vicinity of transcriptional initiation sites of genes is important for gene silencing [14,15]. Detailed analysis of the methylation pattern of human *p16^{INK4a}* gene in bladder cancer cell lines suggests that the critical region for downregulating the promoter activity is located at a nearby transcriptional initiation site, 50–300 bp upstream of the translation initiation site [16].

Recently, 5′ upstream region in rat *p16^{INK4a}* gene has been cloned and demonstrated that a putative TATA box and a transcriptional initiation site in rat p16^{INK4a} promoter are found 390 bp upstream of the translation initiation site, between nt −401 and

*Correspondence to: Department of Orthopedic Surgery, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8522, Japan.

Received 18 June 2003; Revised 10 October 2003; Accepted 22 October 2003

Abbreviations: MFH, malignant fibrous histiocytoma; cdk, cyclin-dependent kinase.

DOI 10.1002/mc.10165

−396, and a transcription start site 10 bp downstream of the box [12]. The detailed location of GpC and CpG sites has also been demonstrated in the promoter region [12].

In this study, we investigated the gene expression of *p16^{INK4a}* and the status of methylation of CpG islands in the p16^{INK4a} promoter region in cell lines recently established in our laboratory; rat lung adenocarcinoma RLCNR from *N*-nitrosobis (2-hydroxypropyl) amine–induced rat lung adenocarcinoma, rat osteosarcoma COS1NR from 4-hydroxy (amino) quinoline 1-oxide–induced osteosarcoma, rat malignant fibrous histiocytoma MFH1NR from 4-hydroxy (amino) quinoline 1-oxide–induced MFH, and normal rat fibroblasts. The data indicated that expression of the rat *p16^{INK4a}* gene was correlated to the methylation status of the promoter region, and especially that the methylation of CpG islands near a putative TATA box region played a critical role for gene silencing of *p16^{INK4a}* gene.

MATERIALS AND METHODS

Cell Culture

The cell lines used in this study were RLCNR from *N*-nitrosobis (2-hydroxypropyl) amine–induced rat lung adenocarcinoma [17], COS1NR from 4-hydroxy (amino) quinoline 1-oxide–induced rat osteosarcoma [18,19], MFH1NR from 4-hydroxy (amino) quinoline 1-oxide–induced rat MFH [19,20], and the rat fibroblast line WLfbt from normal lung tissue (passage 6), all of which were recently established in our laboratory. All cell lines were cultured in Eagle's modified essential medium supplemented with 10% fetal bovine serum in 5% CO_2 atmosphere at 37°C.

Expression of *p16^{INK4a}* mRNA by RT-PCR

Total RNAs were extracted from frozen packed cells with the RNeasy Total RNA system (Qiagen GmbH, Hilden, Germany) and first-strand cDNA was synthesized from 2 μg of samples with the Superscript Preamplification System (Life Technologies, Rockville, MD). All PCR reactions were performed with 0.5 μL of a 10 μL reaction mixture as a template. The primer sequences used for amplification of the rat *p16^{INK4a}* gene were Rp16-a, 5′-AACACTTTCGGTCGTACCC and Rp16-b, 5′-GTCCTCGCAGTTCGAATC [12]. The rat glyceraldehydes-3-phosphate dehydrogenase gene was used as an internal control to adjust the amounts of template. For each gene, multiple cycles of PCR were tested, and the cycle at which the sample having the highest expression reached an amplification plateau was determined; then a cycle number smaller than this was adopted for the analysis.

Bisulfite Sequencing of p16^{INK4a} Promoter Region

Bisulfite treatment of genomic DNA was performed as previously described [12,21]. Genomic DNA was extracted with DNeasy^{TM} tissue kit (Qiagen GmbH) from frozen packed cells of RLCNR, COS1NR, MFH1NR, and WLfbt, and 500 ng of each sample was digested with BamHI restriction enzyme. The digested DNA was denatured in 0.3 N NaOH, then 2.9 M sodium bisulfite (Sigma, St. Louis, MO) and 0.5 mM hydroquinone (Sigma) was added and the mixture underwent 15 cycles of 30 s denaturation at 95°C and 15 min incubation at 50°C. The sample was then desalted with the Wizard DNA cleanup system (Promega, Madison, WI), and desulfonated by treatment with 0.3 N NaOH at room temperature for 5 min. After ethanol precipitation with ammonium acetate, DNA was dissolved in distilled water.

For bisulfite sequencing, PCR was performed with the primer sets, p16-bis-A1, 5′-GTTTGTTGGGAGGAGGAGAGATT and p16-bis-A2, 5′-AAACACCTCTAAAAACACTACTACCC for Region A (nt −478 to −201) and p16-bis-B1, 5′-GTGGGGTGGGTAGTAGTGTT and p16-bis-B2, 5′-ACTAATCTATCTACAAAAAACTCCAT for Region B (nt −233 to 26), all of which were selected from recently cloned rat p16^{INK4a} promoter region (GenBank accession number, AB081658) (Figure 2) [12]. PCR products were cloned into pGEM-T Easy Vector (Promega). Ten clones were sequenced by cycle-sequencing with the BigDye Terminator ready Reaction Mix (PE Applied Biosystems, Foster City, CA) with the primers used for the initial PCR.

5-Aza 2′-Deoxycytidine Treatment for p16^{INK4a} Induction

5-Aza 2′-deoxycytidine (Sigma) was dissolved in phosphate saline buffer. Aliquots were prepared and frozen at −20°C. Exponentially growing cells were treated with 5-aza 2′-deoxycytidine at a concentration of 10 μM for different times (24, 48, and 72 h), and cells then harvested at each time point to analyze changes of p16^{INK4a} expression.

RESULTS AND DISCUSSION

Recently, the 5′ upstream region of rat p16^{INK4a} has been cloned and hypermethylation mediated-gene silencing shown to be well correlated with its methylation status [12]. In this study, we investigated the methylation status and expression of rat p16^{INK4a} with rat lung adenocarcinoma RLCNR, rat osteosarcoma COS1NR and MFH1NR cell lines. RT-PCR analysis demonstrated that RLCNR did not express *p16^{INK4a}* mRNA, while COS1NR and MFH1NR expressed it at levels similar to normal fibroblasts WLfbt (Figure 1). This result indicated that the gene silencing or genomic deletion of the *p16^{INK4a}* gene could be involved in RLCNR. Thus, we further analyzed the methylation status of the promoter region of the *p16^{INK4a}* gene in these tumors. Methylation-specific PCR with primers in the promoter region demonstrated that DNA was methylated in RLCNR, but partially in COS1NR and not methylated in MFH1NR

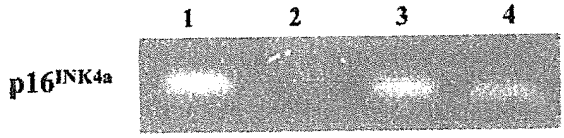

Figure 1. Expression of *p16INK4a* mRNA by RT-PCR. 1, Normal fibroblasts WLfbt; 2, rat lung adenocarcinoma RLCNR; 3, rat osteosarcoma COS1NR; 4, rat malignant fibrous histiocytoma MFH1NR. No *p16INK4a* mRNA was detected in RLCNR, but it was found in COS1NR and MFH1NR at similar levels as WLfbt.

and WLfbt (data not shown), suggesting that the expression of $p16^{INK4a}$ could be related to the methylation status of the promoter regions in these rat malignant tumor cells. It has been reported that the methylation status of CpG islands is important for transcriptional regulation of a gene, especially if the island's location is near a transcriptional start site [13]. A recent study demonstrated that a putative TATA box and a transcriptional initiation site in rat $p16^{INK4a}$ promoter are found 390 bp upstream of the translation initiation site, between nt −401 and −396, and a transcription start site 10 bp downstream of the box [12]. The detailed location of GpC and CpG sites is also demonstrated in the promoter region, and extensive analysis of methylation pattern for rat mammary carcinomas showed that cell line lacking $p16^{INK4a}$ expression is consistently methylated in the flanking region of the putative initiation start site near the TATA box [12]. Therefore, we investigated the detailed methylation status of CpG islands by bisulfite sequencing. The result of bisulfite sequencing is shown in Figure 2, and demonstrates that the CpG islands in Region B (nt −233 to 26) were methylated both in RLCNR and COS1NR, especially highly in COS1NR, whereas those in Region A (nt −478 to −201) containing a

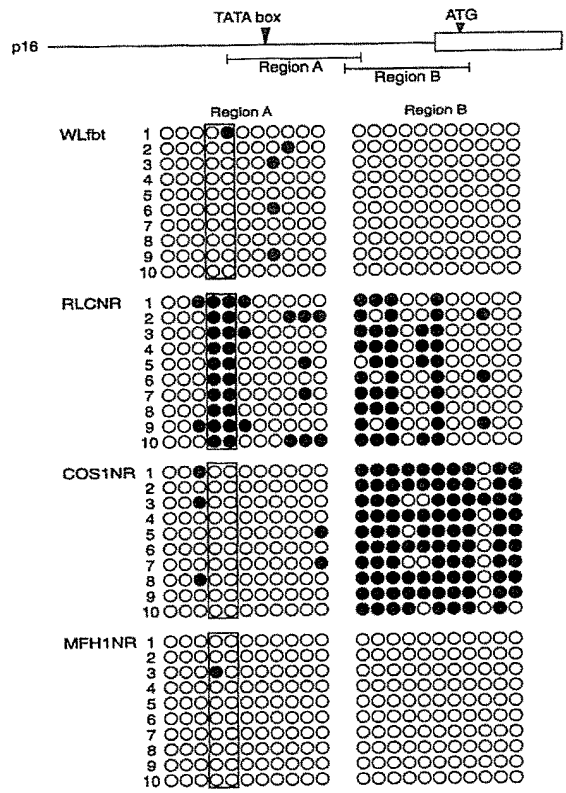

Figure 2. Results of bisulfite sequencing are shown as closed circles (●) for methylated CpG site and open circles (○) for unmethylated CpG site. The CpG islands near the TATA box in Region A, shown as a boxed region, are consistently methylated in RLCNR, but not in other cell lines. The Region B was hypermethylated in COS1NR rather than RLCNR and was not methylated in MFH1NR and WLfbt.

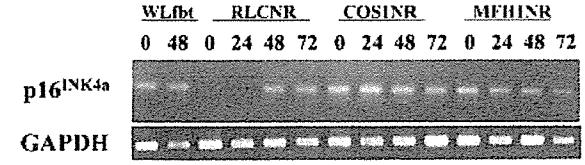

Figure 3. RT-PCR for $p16^{INK4a}$ induction by treatment with 5-aza 2′-deoxycytidine shows the induction of $p16^{INK4a}$ occurring in RLCNR after 48 h treatment. No changes were found in WLfbt, COS1NR, and MFH1NR cell lines.

TATA box were not methylated in COS1NR, in contrast to being methylated in RLCNR. Both regions in MFH1NR and WLfbt were not methylated. Interestingly, the CpG islands near a putative TATA box, located 6 and 10 bp downstream of the box, relevant to the transcription initiation site (shown as a boxed region in Figure 2 and [12]), were consistently methylated in RLCNR, but not in COS1NR and MFH1NR as well as WLfbt, whereas Region B (nt −233 to 26) was hypermethylated in COS1NR rather than in RLCNR.

Loss of transcription could be caused by genomic deletion rather than or in addition to methylation of the promoter region. The treatment with a DNA methyltransferase inhibitor such as 5-aza 2′-deoxycytidine can lead to re-expression of *p16^INK4a*, when gene silencing associated with CpG islands methylation occurs [22]. Therefore, we tested whether expression of mRNA was induced by 5-aza 2′-deoxycytidine on these cells. Our data demonstrated that treatment with 5-aza 2′-deoxycytidine induced the expression of the *p16^INK4a* gene in RLCNR after 48 h, but no changes were observed in COS1NR and MFH1NR, as well as in normal fibroblasts WLfbt (Figure 3). Methylation-specific PCR after 5-aza 2′-deoxycytidine treatment revealed that methylated DNA was converted to unmethylated in RLCNR (data not shown). These results indicated that the methylation-mediated gene silencing made a major contribution to loss of $p16^{INK4a}$ expression in RLCNR rather than genomic deletion.

Although methylation of exon 1α has been used for analysis of $p16^{INK4a}$ involvement in various rat tumors [5,9,10], it has been reported that promoter-region hypermethylation events are critical for losses of key gene functions in neoplastic cells [11,12]. Our data in the present study did not only confirm these previous results, but also provided direct evidence that the methylation of CpG islands near a TATA box and transcription initiation site in the promoter region played a critical role in the gene silencing of the rat *p16^INK4a* gene.

ACKNOWLEDGMENTS

We thank Dr. Kerry Greer for careful reading of the manuscript and English language correction and also Ms. Aya Yamamoto and Mr. Taka-aki Iwamoto for their technical assistance. This work was supported by funds in part by a Grant-in-Aid for Cancer Research from the Ministry of Health, Labor and Welfare of Japan, by a Grant-in Aid for Scientific Research Expenses for Health and Welfare Programs, 2nd-Term Comprehensive 10-Year Strategy for Cancer Control, Cancer Prevention, from the Ministry of Health, Labor and Welfare of Japan, and by a Grant-in-Aid for Scientific Research (C) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES

1. Meyerson M, Enders GH, Wu CL, et al. A family of human cdc2-related protein kinases. EMBO J 1992;11:2909–2917.
2. Sherr CJ, Roberts JM. Inhibitors of mammalian G_1 cyclin-dependent kinases. Genes Dev 1995;9:1149–1163.
3. Ruas M, Peters G. The p16INK4A/CDKN2 tumor suppressor and its relatives. Biochem Biophys Acta 1998;1378:F115–F177.
4. Otterson GA, Kratze RA, Coxon A, Kim YW, Kaye FJ. Absence of $p16^{INK4a}$ protein is restricted to the subset of lung cancer lines that retains wildtype Rb. Oncogene 1994;9:3375–3378.
5. Belinsky SA, Nikula KJ, Palmisano WA, et al. Aberrant methylation of $p16^{INK4a}$ is an early event in lung cancer and a potential biomarker for early diagnosis. Proc Natl Acd Sci USA 1998;5:11891–11896.
6. Wei G, Lonardo F, Ueda T, et al. *CDK4* gene amplification in osteosarcoma: Reciprocal relationship with *INK4A* gene alteration and mapping of 12q13 amplicons. Int J Cancer 1999;80:199–204.
7. Benassi MS, Molendini L, Gamberi G, et al. Alteration of pRb/p16/cdk4 regulation in human osteosarcoma. Int J Cancer 1999;84:489–493.
8. Herman JG, Merlo A, Mao L, et al. Inactivation of the *CDKN2/p16/MTS1* gene is frequently associated with aberrant DNA methylation in all common human cancers. Cancer Res 1995;55:4525–4530.
9. Pulling LC, Klinge DM, Belinsky SA. $p16^{INK4a}$ and β-catenin alterations in rat liver tumors induced by NNK. Carcinogenesis 2001;22:461–466.
10. Tanaka T, Iwasa Y, Kondo S, Hirai H, Toyokuni S. High incidence of allelic loss on chromosome 5 and inactivation of $p15^{INK4B}$ and $p16^{INK4A}$ tumor suppressor genes in oxystress-induced renal cell carcinoma of rats. Oncogene 1999;18:3793–3797.
11. Herman JG, Baylin SB. Promoter-region hypermethylation and gene silencing in human cancer. Curr Top Microbiol Immunol 2000;249:35–54.
12. Abe M, Okochi E, Kuramoto T, et al. Cloning of the 5′ upstream region of the rat *p16* gene and its role in silencing. Jpn J Cancer Res 2002;93:1100–1106.
13. Chan MF, Liang G, Jones PA. Relationship between transcription and DNA methylation. Curr Top Microbiol Immunol 2000;249:75–86.

14. Pfeifer GP, Steigerwald SD, Hansen RS, Gartler SM, Riggs AD. Polymerase chain reaction-aided genomic sequencing of an X chromosome-linked CpG island: Methylation patterns suggest clonal inheritance, CpG site autonomy, and an explanation of activity state stability. Proc Natl Acad Sci USA 1990;87:8252–8256.
15. Levine A, Cantoni GL, Razin A. Methylation in the preinitiation domain suppresses gene transcription by an indirect mechanism. Pro Natl Acad Sci USA 1992;89: 10119–10123.
16. Gonzalgo ML, Hayashida T, Bender CM, et al. The role of DNA methylation in expression of the p19/p16 locus in human bladder cancer cell lines. Cancer Res 1998;58:1245–1252.
17. Tsujiuchi T, Tsutsumi M, Sasaki Y, Murata N, Konishi Y. Mutations of adenomatous polyposis coli and β-catenin genes during progression of lung tumors induce by *N*-nitrosobis (2-hydroxypropyl) amine in rats. Cancer Res 2000; 60:6611–6616.
18. Honoki K, Tsutsumi M, Miyauchi Y, et al. Increased expression of nucleoside diphosphate kinase/nm23 and c-Ha-ras mRNA is associated with spontaneous lung metastasis in rat-transplantable osteosarcomas. Cancer Res 1993;53: 5038–5042.
19. Honoki K, Tsujiuchi T, Sasaki Y, et al. Differential expression of cytokines in rat osteosarcoma and malignant fibrous histiocytoma cell lines induced by 4-(hydroxyamino) quinoline-1-oxide. Mol Carcinogenesis 2002;33:81–87.
20. Konishi Y, Maruyama H, Mii Y, Miyauchi Y, Yokose Y, Masuhara K. Malignant fibrous histiocytomas induced by 4-(hydroxyamino) quinoline-1-oxide in rats. J Natl Cancer Inst 1982;68:859–862.
21. Rein T, Zorbas H, Depaphilis ML. Active mammalian replication origins are associated with a high-density cluster of mCpG dinucleotides. Mol Cell Biol 1997;17:416–426.
22. Zhu W-G, Dai Z, Ding H, et al. Increased expression of unmethylated CDK2D by 5-aza-2′-deoxycytidine in human lung cancer cells. Oncogene 2001;20:7787–7796.

MOLECULAR CARCINOGENESIS 39:199–205 (2004)

ARTICLES

Alterations of the M6p/Igf2 Receptor Gene in Hepatocellular Carcinomas Induced by *N*-Nitrosodiethylamine and a Choline-Deficient L-Amino Acid–Defined Diet in Rats

Toshifumi Tsujiuchi,* Yasutaka Sasaki, Yasuo Oka, Hiroki Kuniyasu, Yoichi Konishi, and Masahiro Tsutsumi

Department of Oncological Pathology, Cancer Center, Nara Medical University, Nara, Japan

To elucidate whether the M6p/Igf2 receptor (M6p/Igf2r) gene might be involved in exogenous and endogenous liver carcinogenesis, we investigated its alteration in hepatocellular carcinomas (HCCs) induced by *N*-nitrosodiethylamine (DEN) and by a choline-deficient L-amino acid-defined (CDAA) diet in rats. Male F344 rats, 6 wk old, received a single intraperitoneal (i.p.) injection of DEN at a dose of 10 mg/kg body weight, followed by combined treatment with partial hepatectomy and colchicine to induce cell cycle disturbance, and a selection procedure regimen, HCCs being obtained after 42 wk. With continuous CDAA diet feeding, tumors were sampled after 75 wk. Total RNA was extracted from individual HCCs for assessment of mutations within exons 27, 28, 31, 33, and 34, and aberrant transcript of the *M6p/Igf2r* gene by reverse transcription (RT)-polymerase chain reaction (PCR)-single strand conformation polymorphism (SSCP) and RT-PCR analyses, respectively. Mutations were detected in three of 15 HCCs (20%) induced by the CDAA diet, a TTT to TTG (Phe to Leu) transversion at codon 1516 and two AAG to AGG (Lys to Arg) transitions at codon 1620, but in none of those caused by DEN. Aberrant transcripts were found in seven of 15 HCCs after DEN treatment (46.7%) and in two of 15 HCCs induced by the CDAA diet (13.3%). These results suggest that alterations of the *M6p/Igf2r* gene may be involved in both exogenous and endogenous liver carcinogenesis with the different patterns and frequencies.

Key words: M6p/Igf2r; hepatocellular carcinoma; choline-deficient L-amino acid-defined diet, nitrosamine; rat

INTRODUCTION

Liver carcinogenesis can be divided into two categories: that induced by exogenous carcinogens, and that due to endogenous changes that occur without any established carcinogen exposure. *N*-nitrosodiethylamine (DEN) is one of the best-known liver carcinogens in rats. We have reported that a cell-cycle disturbance induced in DEN-initiated hepatocytes by colchicine gives a growth advantage to putative preneoplastic lesions under conditions of partial hepatectomy and selection pressure, so that a high incidence of hepatocellular carcinomas (HCCs) can be obtained within a short latent period [1,2]. We have also demonstrated high yields of HCCs associated with cirrhosis caused by chronic administration of a CDAA diet that does not contain any known carcinogens [3]. Since our studies revealed different effects of chemopreventive agents [4] and variation in genetic alterations [5] in our two liver models, there is a possibility that different mechanisms underlie exogenous and endogenous hepatocarcinogenesis in rats.

The mannose 6-phosphate/insuline-like growth factor-II receptor (M6P/IGF2R) is a multifunctional cell membrane-associated glycoprotein that plays a critical role in regulating cell growth by facilitating the activation of the potent growth inhibitor, transforming growth factor β (TGF-β) [6], and degrading the growth stimulator IGF2 [7,8]. The functional loss of this receptor is therefore predicted both to increase cell proliferation and reduce apoptosis, consistent with the *M6P/IGF2R* gene functioning as a tumor suppressor. Recently, alterations of the *M6P/IGF2R* gene have been reported in several human tumors, including liver, endometrial, stomach, colorectal, lung, and head and neck cancers [9–13]. In the rat, a

*Correspondence to: Department of Oncological Pathology, Cancer Center, Nara Medical University, Kashihara, Nara 634-8521, Japan.

Received 18 September 2003; Accepted 30 October 2003

Abbreviations: M6p/Igf2r, mannose 6-phosphate/insulin-like growth factor II receptor; DEN, *N*-nitrosodiethylamine; CDAA diet, choline-deficient L-amino acid-defined diet; HCC, hepatocellular carcinoma; RT, reverse transcription; PCR, polymerase chain reaction; SSCP, single-strand conformation polymorphism; TGF β, transforming growth factor β; nt, nucleotides.

DOI 10.1002/mc.20010

high frequency of alterations of the *M6p/Igf2r* gene has been detected in HCCs induced by DEN [14]. Previously, we have reported that genes related to the TGF-β signaling pathway, such as Smad2, Smad4, and TGF-β receptor II, might be altered in HCCs induced by the CDAA diet, but not by DEN [15]. In the present study, to assess further whether differences between exogenous and endogenous liver carcinogenesis may exist in the TGF-β signaling pathway, we investigated alterations of the *M6p/Igf2r* gene in HCCs induced by two inducing regimens in rats.

MATERIALS AND METHODS

Animals

A total of 33 male Fischer-344 rats, 5 wk old, was purchased from Japan SLC, Inc. (Shizuoka, Japan) and housed 3–5 rats in a plastic cage with white flake bedding in an air-conditioned room, with a constant temperature of 25°C and a 12-h light-dark cycle. Food and water were given ad libitum. After a 1-wk acclimation period on a basal diet in pellet form (CF-2 Diet; Clea Japan, Tokyo, Japan), the animals were allocated to the experimental groups.

Chemicals and Diets

DEN was purchased from Wako Pure Chemical Co. Ltd. (Kyoto, Japan) and diluted with a 0.9% NaCl solution to a concentration of 0.1%. Colchicine was purchased from Sigma Chemical Co. (St. Louis, MO) and dissolved in a 0.9% NaCl solution to a concentration of 0.05%. Carbon tetrachloride was purchased from Nacalai Tesque, Inc. (Kyoto, Japan) and diluted 1:1 with corn oil. AAF (Nacalai) was admixed into Oriental MF powdered basal diet at a concentration of 0.02%. The CDAA diet, with the composition described previously [3,16], was purchased from Dyets, Inc. (Bethlehem, PA) and stored at 4°C immediately upon arrival.

Animal Treatments

With the exogenous agent, the method for the production of HCCs was as previously described [1,2]. Fifteen animals received DEN at a single intraperitoneal (i.p.) dose of 10 mg/kg body weight and 4 h thereafter partially hepatectomized by the method of Higgins and Anderson [17]. Colchicine at an i.p. dose of 0.5 mg/kg body weight was administered two times, one and three days after DEN. After an 11-d recovery period, rats were placed on the selection procedure comprising diet containing AAF at a concentration of 0.02% for 2 wk and a single intragastric administration of carbon tetrachloride at 1 mL/kg body weight at the midpoint [18]. All rats were killed by exsanguination from the abdominal aorta under light ether anesthesia 42 wk after the beginning of the experiment. For endogenous carcinogenesis, 15 animals were continuously given the CDAA diet and similarly killed 75 wk after the beginning of the experiment. Three animals were maintained on basal diet and free from exposure to the carcinogen throughout the experimental period in order to obtain normal liver tissue.

Liver Samples

At death, the livers were immediately excised, and grossly apparent tumors were dissected with careful elimination of their surrounding tissues. Portions of the tumors were taken, fixed in 10% neutralized formalin, processed routinely for production of paraffin sections, stained with hematoxylin and eosin (H&E), and histologically evaluated. Remaining tumor samples were immediately frozen in liquid nitrogen and stored at −80°C until use.

Reverse Transcription (RT)-Polymerase Chain Reaction (PCR)–Single-Strand Conformation Polymorphism (SSCP) Analysis of the *M6p/Igf2r* Gene

Total RNA was extracted from frozen tissue using ISOGEN (Nippon Gene, Inc., Toyama, Japan) and first-strand cDNA was synthesized from 5-μg samples with Ready-To-Go Your-Prime First-Strand Beads (Pharmacia Co. Ltd., Tokyo, Japan). To eliminate possible false positives caused by residual genomic DNA, all samples were treated with DNase.

RT-PCR–SSCP analysis was carried out as previously described [19], using the primers listed in Table 1. All were designed from the rat M6p/Igf2r

Table 1. Primer Sequences of the *M6p/Igf2r* Gene Used in This Study

cDNA location	Primer	Size of amplified product (bp)	Annealing temperature (°C)
nt 3576–3816	IGF2R-1F: 5′-CAACTGTGAGTATGTGTTTG-3′ IGF2R-1R: 5′-CTGGCATGATGAGACTGCCT-3′	241	56
nt 3767–4005	IGF2R-2F: 5′-TAGATGTGTGCTCTGCCCAT-3′ IGF2R-2R: 5′-AGTTTCCTTCAGAAACACTGG-3′	239	59
nt 4178–4447	IGF2R-3F: 5′-TCATCAACGTATGCAAGTCT-3′ IGF2R-3R: 5′-AGTCCTGTACAGCACTGATG-3′	270	59
nt 4504–4749	IGF2R-4F: 5′-CACGATGACTGCCAAGTCACC-3′ IGF2R-4R: 5′-ATTCTCATACACCAGCTGCAAG-3′	246	63
nt 4706–4937	IGF2R-5F: 5′-GGCTGAGCTACAAGGATCAGGT-3′ IGF2R-5R: 5′-CCATTCCGCACGGTGCATTCCG-3′	232	66

cDNA sequence (GenBank accession no: U59809). The PCR amplification was performed in 10 μL of reaction mixture consisting of 1 μM each primer, 200 μM each dNTP, 1× PCR buffer (Perkin Elmer, Applied Biosystems Division, Foster City, CA), 68 nM [α-^{32}P] dCTP, 2.5 U of Ampli Taq (Perkin Elmer), and 0.5 μL of synthesized cDNA mixture under the following reaction conditions: a denaturation step for 5 min at 95°C; 35 cycles of 1 min at 95°C; 1 min at 56, 59, 63, and 66°C, and 2 min at 72°C; and a final extension for 10 min at 72°C. PCR products were diluted with 90 μL of loading solution containing 90% formide, 20 mM EDTA, and 0.05% xylen cyanol and bromophenol blue, denatured at 90°C for 2 min and applied to 5, 6, 8, and 10% polyacrylamide gels containing 0.5× Tris-borate EDTA buffer with or without 5% glycerol. Electrophoresis was performed at 40 W for about 2.5 h at 30°C. Gels were dried on filter paper and used to expose X-ray films at −80°C.

RT-PCR Amplification

The cDNAs synthesized from frozen tissues were assessed for aberrant splicing of the *M6p/Igf2r* gene by nested RT-PCR analysis [19], using the primers; 1F: 5′-CTGGGAACGAGTACGACCTGAGTGC-3′, 1R: 5′-TGCTGCT CTCAAAGTTCAGGTAGAC-3′, 2F: 5′-CTGCTGTGGACACGTCTGTGCAT-3′, and 2R: 5′-CTCATTGGCCACGGGATTGAATAT-3′ designed according to the rat M6p/Igf2r cDNA sequence. The first round of PCR amplification was performed in 10 μL of reaction mixture consisting of 1 μM primers 1F and 1R, 200 μM each dNTP, 1× PCR buffer (Perkin Elmer), 2.5 U of Ampli Taq (Perkin Elmer), and 0.5 μL of synthesized cDNA mixture under the following reaction conditions: a primary denaturation step for 2 min at 95°C; 30 cycles of 30 s denaturation at 95°C; 30 s annealing at 66°C, and 1 min extension at 72°C; and a final extension for 10 min at 72°C. The amplified product was diluted 50-fold in TE buffer, and 1 μL of the dilutant was subjected to the second round of PCR amplification with the above conditions except that the annealing temperature was 70°C, using primers 2F and 2R. PCR products were then separated on 2% NuSieve agarose gels (BMA, Rockland, ME) containing 0.05 μg/mL ethidium bromide. Each nested RT-PCR assay was repeated at least twice for confirmation using the originally extracted RNA.

DNA Nucleotide Sequencing

Following the RT-PCR–SSCP and RT-PCR analyses, DNA fragments from abnormal shift bands and aberrant splicing bands in the gels were extracted and reamplified. The obtained PCR products were directly sequenced using a BigDye terminator v3.0 cycle sequencing ready reaction kit (Applied Biosystems Japan Ltd., Tokyo, Japan) and an ABI PRISM 310 genetic analyzer (Applied Biosystems Japan Ltd.). To confirm the results, each PCR product was sequenced with the forward and reverse primers at least twice.

RESULTS

The 15 HCCs induced by DEN in 15 rats and 15 HCCs induced by the CDAA diet in 15 other rats used for the analysis were all histologically well-differentiated carcinomas.

Representative results of RT-PCR–SSCP and sequencing analyses are shown in Figure 1A and B. Three out of 15 HCCs induced by the CDAA diet showed abnormal band shifts in the regions of nt 4504–4749 and nt 4706–4937, indicative of mutations (20%) (Figure 1A). These were established to be a TTT to TTG (Phe to Leu) transversion at codon 1516 and two AAG to AGG (Lys to Arg) transitions at codon 1620 (Figure 1B). The sequencing analysis was confirmed by using with the forward sequenced primer. The 15 HCCs induced by DEN showed no abnormal band shifts for M6p/Igf2r (Table 2).

The nested RT-PCR analysis revealed that, whereas only the normal product band was amplified at 1898 bp in all three normal liver tissues, in the region of nt 3287–5184, one abnormal-sized band additionally appeared representing aberrant RT-PCR products in seven of 15 HCCs induced by DEN (46.7%) and in two of 15 HCCs induced by the CDAA diet (13.3%). Representative RT-PCR results are shown in Figure 2A. Such abnormal-sized transcripts were seen at six different positions of 1004, 971, 821, 739, 695, and 404 bp in HCCs induced by DEN and at two of 1019 and 635 bp in HCCs induced by the CDAA diet. Sequencing analysis revealed these aberrant fragments to be due to absence in the regions of nt 3956–4849, 4234–5160, 3964–5040, 3878–5036, 3700–4902, and 3393–4886 in HCCs induced by DEN, and in the regions of nt 3818–4696 and nt 3482–4744 in HCCs induced by the CDAA diet (Figure 2B). Normal-sized transcripts demonstrated normal sequences, except with CDAA 2, 7, and 15 (data not shown) (Table 3).

DISCUSSION

Inactivation of the *M6P/IGF2R* gene has been demonstrated in a number of human cancers. LOH has been reported to occur in 60% of HCCs and 30% of breast malignancies, with mutations located in exons 27, 28, 31, 33, and 34 also identified in the remaining allele [9,12,20–22]. The affected exons encode the IGF2 and M6P binding domains, which are functionally important regions of M6P/IGF2R [8,23–25]. In an early study, no mutations were detected in these exons in HCCs induced by DEN, except for a single mutation at the splicing site [14]. In the present investigation, we also found no mutations in HCCs induced by DEN within exons 27, 28, 31, 33, and 34. However, we did detect missense mutations in exons 33 and 34, which encode the IGF2 binding domain [8,23–25], in HCCs

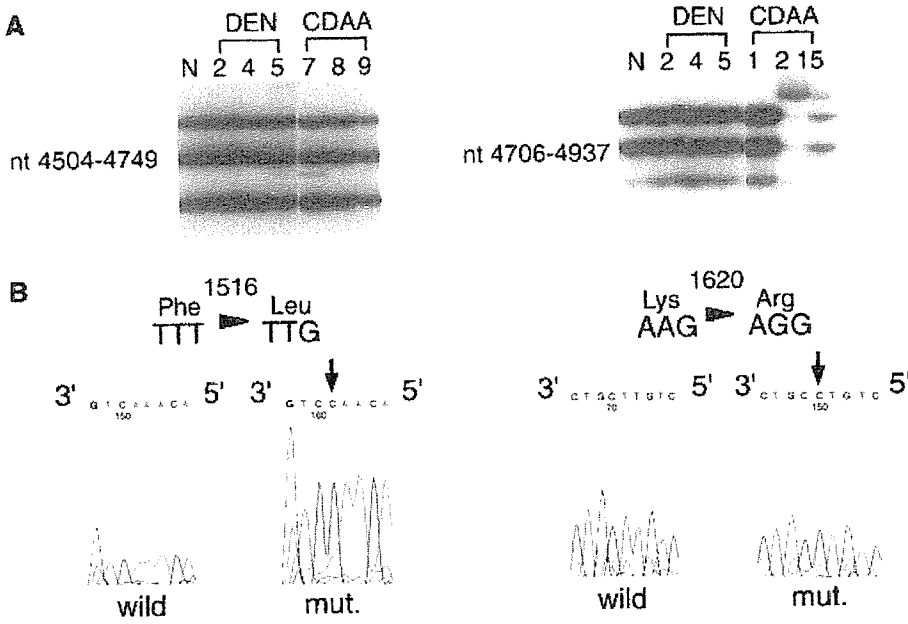

Figure 1. Representative results of RT-PCR–SSCP analysis for mutations of the *M6p/Igf2r* gene (A) CDAA2 demonstrated a band shift in the regions of nt 4504–4749 of M6p/Igf2r and CDAAs 2 and 15 in the regions of nt 4705–4937. N, normal liver. (B) The mutation pattern of the M6p/Igf2r detected by sequencing analysis. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

induced by the CDAA diet. Previously, we reported oxidative damage to liver DNA and extra-DNA subhepatocellular components due to reactive oxygen species in animals fed with the CDAA diet [16,26]. Oxidative damage to liver DNA, as evidenced by 8-hydroxydeoxyguanine formation, can be detected as early as one day following CDAA diet administration and progressively accumulates at least up to day 84 [16,26]; such feeding induces specific G/C-to-T/A and A/T-to-C/G transversions [27]. Moreover, the T/A-to-C/G transition was shown to be a common type of mitochondrial DNA mutation in colorectal tumors; this may be related to a high level of reactive oxygen species [28]. The M6p/Igf2r mutations in HCCs induced by the CDAA diet were A/T-to-G/C transitions and a T/A-to-G/C transversion, and therefore, might have been due to oxygen species generated during endogenous liver carcinogenesis.

Aberrant transcripts of the *M6p/Igf2r* gene have been detected in rat HCCs induced by DEN [14]. Most of them are associated with deletions in the regions of the M6P/IGF2R that contains the IGF2 and M6P binding domains, including exons 27 to 35 [8,14,23,24]. In the present study, aberrant transcripts were found in 7 HCCs induced by DEN and in 2 HCCs induced by the CDAA diet and the deletions were due to the absence in exons 27 to 34. It seems that these regions of the M6P/IGF2R may be more sensitive to chemical carcinogen action than to endogenous factors, such as oxidative DNA damage. Recently, we have reported aberrant transcripts of M6p/Igf2r in rat lung adenocarcinomas induced by *N*-nitrosobis(2-hydroxypropyl)amine, with no mutations [19]. The M6p/Igf2r is imprinted in mice and rats with only the maternal allele being transcribed [13,29], while this gene is not imprinted in humans, since imprinting at this locus was lost in Eutherian mammals approximately 75 million years ago [29,30]. Therefore, it is hypothesized that alteration of one allele might be enough to inactivate the M6p/Igf2r in mice and rats. In fact, aberrant transcripts

Table 2. Results for *M6p/Igf2r* Gene Mutations in Rat HCCs

Sample no.	Exon	Nucleotide	Codon change	Amino acid substitution
CDAA2	34	4859	1620 AAG to AGG	Lys to Arg
CDAA7	33	4548	1516 TTT to TTG	Phe to Leu
CDAA15	34	4859	1620 AAG to AGG	Lys to Arg

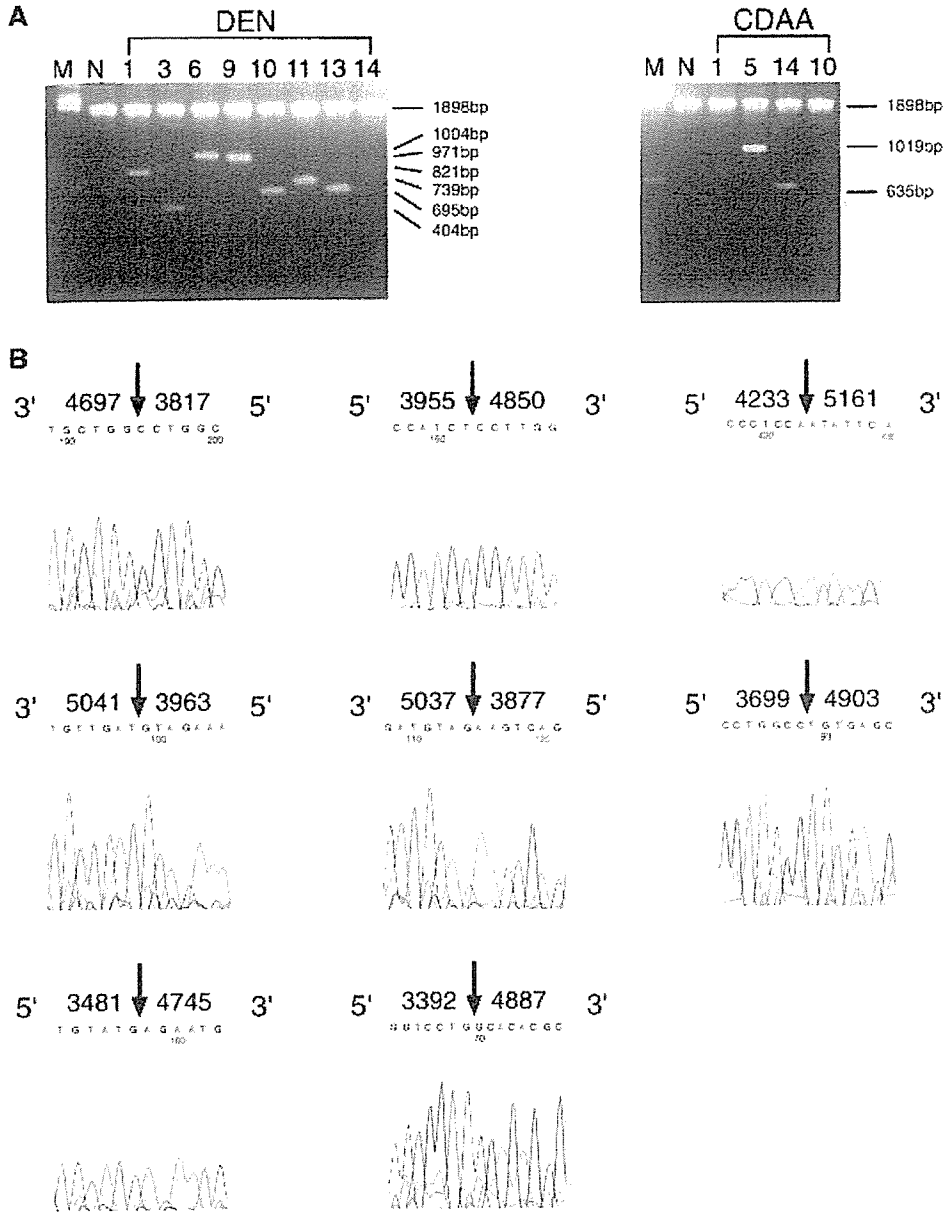

Figure 2. Aberrant transcription of the *M6p/Igf2r* gene. (A) Representative results of the nested RT-PCR analysis. M, size markers; N, normal liver. (B) Patterns of aberrant transcripts. Arrows indicate the junction between nt 3817 and 4697 (CDAA5), 3955 and 4850 (DEN6), 4233 and 5161 (DEN9), 3963 and 5041 (DEN1), 3877 and 5037 (DEN11), 3699 and 4903 (DEN10, 13), 3481 and 4745 (CDAA14), and 3392 and 4887 (DEN3) in the aberrant transcripts.

were found from the expressed allele in rat HCCs induced by DEN, suggesting imprinted M6p/Igf2r to represent a susceptibility locus for chemical carcinogens in mice and rats more than in man [14]. In the present study, normal-sized bands also appeared with mutated and aberrant transcribed cases but these could have arisen from contamination with surrounding normal liver tissues.

In conclusion, the present study indicated that alterations of the *M6p/Igf2r* gene may play roles in

Table 3. Results for Aberrant Transcripts From the *M6p/Igf2r* Gene in Rat HCCs

Sample no.	cDNA alterations
DEN1	1077 bp deletion (nt 3964–5040)
DEN3	1494 bp deletion (nt 3393–4886)
DEN6	894 bp deletion (nt 3956–4849)
DEN9	927 bp deletion (nt 4234–5160)
DEN10	1203 bp deletion (nt 3700–4902)
DEN11	1159 bp deletion (nt 3878–5036)
DEN13	1203 bp deletion (nt 3700–4902)
CDAA5	879 bp deletion (nt 3818–4696)
CDAA14	1263 bp deletion (nt 3482–4744)

both exogenous and endogenous liver carcinogenesis with different patterns and frequencies. Together with our previous results [15], the findings suggest that disturbance of the TGF-β signaling pathway may be due to effects of exogenous and endogenous liver carcinogenesis on different genes in rats.

ACKNOWLEDGMENTS

This work was supported in part by Grants-in-Aid for Cancer Research and for Scientific Research Expenses for Health and Welfare Programs, 2nd-Term Comprehensive 10-Year Strategy for Cancer Control, Cancer Prevention, both from the Ministry of Health, Labor and Welfare of Japan. We express our appreciation to Sachiko Nakai and Megumi Yamaguchi for their technical assistance.

REFERENCES

1. Ohashi K, Tsutsumi M, Tsujiuchi T, et al. Enhancement of *N*-nitrosodiethylamine-initiated hepatocellular carcinogenesis caused by a colchicine-induced cell cycle disturbance in partially hepatectomized rats. Cancer Res 1996;56:3473–3479.
2. Tsutsumi M, Ohashi K, Tsujiuchi T, et al. Disturbance of the cell cycle with colchicine enhances the growth advantage of diethylnirosamine-initiated hepatocytes in rats. Jpn J Cancer Res 1996;87:5–9.
3. Nakae D, Yoshiji H, Mizumoto Y, et al. High incidence of hepatocellular carcinomas induced by a choline deficient L-amino acid defined diet in rats. Cancer Res 1992;52:5042–5045.
4. Kobayashi Y, Nakae D, Akai H, et al. Prevention by 1′-acetoxychavicol acetate of the induction but not growth off putative preneoplastic, glutathione *S*-transferase placental form-positive focal lesions in the livers of rats fed a choline-deficient, L-amino acid-defined diet. Carcinogenesis 1998; 19:1809–1814.
5. Tsujiuchi T, Tsutsumi M, Sasaki Y, Takahama M, Konishi Y. Different frequencies and patterns of β-catenin mutations in hepatocellular carcinomas induced by *N*-nitrosodiethylamine and a choline deficient L-amino acid defined diet in rats. Cancer Res 1999;59:3904–3907.
6. Dennis P, Rifkin D. Cellular activation of latent transforming growth factor β requires binding to the cation-independent mannose 6-phosphate/insuline-like growth factor type II receptor. Proc Natl Acad Sci USA 1991;88:580–584.
7. Morgan D, Edman J, Standring D, et al. Insulin-like growth factor II receptor as a multifunctional binding protein. Nature 1987;329:301–307.
8. Kornfeld S. Structure and function of the mannose 6-phosphate/insulin-like growth factor II receptors. Ann Rev Biochem 1992;61:307–330.
9. DeSouza AT, Hankins GR, Washington MK, Orton TC, Jirtle RL. *M6P/IGF2R* gene is mutated in human hepatocellular carcinomas with loss of heterozygosity. Nature Genet 1995; 11:447–449.
10. Ouyang H, Shiwaku O, Hagiwara H, et al. The insulin-like growth factor II receptor gene is mutated in genetically unstable cancers of the endometrium, stomach and colorectum. Cancer Res 1997;57:1851–1854.
11. Souza RF, Appel R, Yin J, et al. Microsatellite instability in the insulin-like growth factor II receptor gene in gastrointestinal tumors. Nat Genet 1996;14:255–257.
12. Kong F-M, Anscher MS, Washington MK, Killian JK, Jirtle RL. M6P/IGF2R is mutated insquamous cell carcinoma of the lung. Oncogene 2000;19:1572–1578.
13. Jamieson TA, Brizel DM, Killia JK, et al. M6P/IGF2R loss of heterozygosity in head and neck cancer associated with poor patient prognosis. BMC Cancer 2003;13:4.
14. Mills JJ, Falls JG, De Souza AT, Jirtle RL. Imprinted M6p/Igf2 receptor is mutated in rat liver tumors. Oncogene 1998;16: 2797–2802.
15. Sasaki Y, Tsujiuchi T, Murata N, Tsutsumi M, Konishi Y. Alterations of the transforming growth factor-β signaling pathway in hepatocellular carcinomas induced endogenously and exogenously in rats. Jpn J Cancer Res 2001;92: 16–22.
16. Nakae D, Yoshiji H, Maruyama H, Kinugasa T, Denda A, Konishi Y. Production of both 8-hydroxydeoxyguanosine in liver DNA and γ-glutamyltransferase-positive hepatocellular lesions in rats given a choline-deficient L-amino acid-defined diet. Jpn J Cancer Res 1990;81:1081–1084.
17. Higgins GM, Anderson RM. Experimental pathology of the liver. 1. Restration of the liver of the white rat following partial surgical removal. Arch Pathol Lab Med 1931;12: 1186–1202.
18. Cayama E, Tsuda H, Sarma DS, Farber E. Initiation of chemical carcinogenesis requires cell proliferation. Nature 1978;275: 60–62.
19. Tsujiuchi T, Sasaki Y, Tsutsumi M, Konishi Y. Alterations of the M6p/Igf2 receptor gene in lung adenocarcinomas induced by *N*-nitrosobis(2-hydroxypropyl)amine in rats. Mol Carcinog 2003;36:32–37.
20. De Souza AT, Hankins GR, Washington MK, Fine RL, Orton TC, Jirtle RL. Frequent loss of heterozygosity on 6q at the mannose-6-phosphate/insulin-like growth factor II receptor locus in human hepatocellular tumors. Oncogene 1995;10: 1725–1729.
21. Yamada T, De Souza AT, Finkelstein S, Jirtle RL. Loss of the gene encoding mannose 6-phosphate/insulin-like growth factor II receptor is an early event in liver carcinogensis. Proc Natl Acad Sci USA 1997;94:10351–10355.
22. Hankins GR, De Souza AT, Bentley RC, et al. M6P/IGF2 receptor: A candidate breast tumor suppressor gene. Oncogene 1996;12:2003–2009.
23. Dahms NM. Insulin-like growth factor II/cation-independent mannose 6-phosphate receptor and lysosomal enzyme recognition. Biochem Sci Trans 1996;24:136–141.
24. Roberts DL, Weix DJ, Dahms NM, Kim J-JP. Molecular basis of lysosomal enzyme recognition: Three-dimensional structure of the cation-dependent mannose 6-phosphate receptor. Cell 1998;93:639–648.
25. Hass AB. Keys to the hidden treasures of the mannose 6-phosphate/insuline-like growth factor 2 receptor. Am J Pathol 2003;162:3–6.
26. Yoshiji H, Nakae D, Mizumoto Y, et al. Inhibitory effect of dietary iron deficiency on inductions of putative preneoplastic lesions as well as 8-hydroxydeoxyguanosine in DNA and lipid peroxidation in livers of rats caused by exposure to a choline-deficient L-amino acid defined diet. Carcinogenesis 1992;13:1227–1233.

27. Cheng KC, Cahill DS, Kasai H, Nishimura S, Loeb LA. 8-Hydroxydeoxyguanosine, an abundant form of oxidative DNA damage, cause G → T and A → C substitutions. J Biol Chem 1992;267:166–172.
28. Polyak K, Li Y, Zhu H, et al. Somatic mutation of the mitocchondrial genome in human colorectal tumors. Nat Genet 1998;20:291–293.
29. Kalscheuer VM, Mariman EC, Schepens MT, Rehder H, Ropers H-H. The insulin-like growth factor type-2 receptor gene is imprinted in the mouse but not in humans. Nat Genet 1993;5:74–78.
30. Killian JK, Nolan CM, Wylie AA, et al. Divergent evolution in M6P/IGF2R imprinting from the Jurassic to the Quaternary. Hum Mol Genet 2001;10:1721–1728.

MOLECULAR CARCINOGENESIS 40:241–246 (2004)

Alterations of the *Dutt1/Robo1* Gene in Lung Adenocarcinomas Induced by *N*-Nitrosobis(2-Hydroxypropyl)Amine in Rats

Toshifumi Tsujiuchi,* Yasutaka Sasaki, Yasuo Oka, Hiroki Kuniyasu, and Masahiro Tsutsumi

Department of Oncological Pathology, Cancer Center, Nara Medical University, Kashihara, Nara, Japan

Abnormalities of tumor suppressor genes (TSGs) on chromosome 3p are known to be important for the development of human lung cancers. In the present study, we investigated alterations of the *Dutt1/Robo1* gene, as a possible tumor suppressor in this region, in rat lung adenocarcinomas induced by *N*-nitrosobis(2-hydroxypropyl)amine (BHP). Male Wistar rats, 6-wk-old, were given 2000 ppm BHP in their drinking water for 12 wk and maintained without further treatment until killed at wk 25. A total of 12 lung adenocarcinomas were obtained and total RNAs were extracted from each for assessment of aberrant transcripts of the *Dutt1/Robo1* gene by reverse transcription (RT)-polymerase chain reaction (PCR) analysis. Aberrant transcripts bearing deletions of nucleotides (nt) 55–4318, 89–4346, 605–4221, and 929–4318 were detected in four of 12 adenocarcinomas (33.3%). Loss or reduced expression of the *Dutt1/Robo1* gene was not found in any of the adenocarcinomas. Genomic DNAs extracted from six adenocarcinomas for Southern blot analysis did not show any evidence of deletion or gross rearrangement of the *Dutt1/Robo1* gene. These results suggest that alterations of the *Dutt1/Robo1* gene may be involved in the development of some lung adenocarcinomas induced by BHP in rats.

Key words: *Dutt1/Robo1*; lung adenocarcinoma; rat; nitrosamine

INTRODUCTION

Deletion of the chromosome 3p is frequently detected in a wide range of solid tumors, suggesting the presence of tumor suppressor genes (TSGs) in this region [1–6]. The several genes have been identified as candidates with possible functional roles in tumor pathogenesis [5,6], but it is still obscure which are the key players in lung carcinogenesis. A human homologue of the *Drosophila* Roundabout gene *DUTT1/ROBO1*, isolated from the U2020 region at 3p12 [7], has the domain structure of the neural-cell adhesion molecules superfamily and is widely expressed, being implicated in the guidance and migration of axons, myoblasts, and leukocytes in vertebrates [8,9]. Recently, it has been reported that loss of DUTT1/ROBO1 expression and hypermethylation of CpG sites within the promoter region may occur in human cancers, such as examples occurring in the lung, breast, and kidney [10]. The *DUTT1/ROBO1* gene thus has been considered as a candidate TSG.

Lung cancer is one of the most common human malignancies, but the rate limiting molecular events for its development are still largely unknown. Recently, it has been demonstrated that 3p genetic alterations appear early in the pathogenesis of lung cancer being found in the smoking-damaged respiratory system, including histologically normal as well as preneoplastic epithelium [11–13]. Because tobacco smoke contains several chemicals, such as nitrosamines [14], obviously capable of causing lung cancers, it can be hypothesized that they induce the 3p genetic alterations. For example, the *FHIT* gene located at 3p14.2 is frequently altered in lung cancers and may be a target of chemical carcinogens such as nitroso-compounds found in tobacco smoke [15–18]. Our recent study indicated that FHIT alterations arise in rat lung adenocarcinomas induced by *N*-nitrosobis(2-hydroxypropyl)amine (BHP), supporting this hypothesis [19]. In the present study, to assess whether another possible TSG in this region may be another target, we investigated alterations of the *Dutt1/Robo1* gene in rat lung adenocarcinomas.

The experimental model used in this study features development of nonsmall cell lung cancers (NSCLCs) in rats given BHP in their drinking water, with high yields of adenomatous lesions, including adenocarcinomas [20,21]. Because the step by step development of lung malignancies is accessible with

Abbreviations: TSG, tumor suppressor gene; BHP, *N*-nitrosobis(2-hydroxypropyl)amine; NSCLC, nonsmall cell lung cancer; PCR, polymerase chain reaction; RT, reverse transcription; nt, nucleotides; SCLC, small cell lung cancer.

*Correspondence to: Laboratory of Cancer Biology and Bioinformatics, Department of Life Science, Faculty of Science and Technology, Kinki University, 3-4-1 Kowakae, Higashiosaka, Osaka 577-8502, Japan.

Received 9 June 2003; Revised 4 February 2004; Accepted 21 May 2004

DOI 10.1002/mc.20037

this model, molecular mechanisms involved can readily be investigated. So far, we have detected a high frequency of mutations of Ki-ras, adenomatous polyposis coli, and β-catenin genes, but not *Ha-ras* and *p53* genes in NSCLCs as well as their preneoplastic lesions [22,23]. Alterations of TGFβ signaling pathway associated genes, such as those encoding the mannose 6-phosphate/insulin-like growth factor II receptor, TGFβ receptor II, Smad2, and Smad4 in lung adenocarcinomas have also been found [24–26].

MATERIALS AND METHODS

Animals and Treatment

A total of 15 male Wistar rats were purchased at 5 wk of age from Japan SLC, Inc. (Shizuoka, Japan) and housed three to five in a plastic cage with white flake bedding in an air-conditioned room, with a constant temperature of 25°C and a 12-h light-dark cycle. Food and water were given ad libitum throughout the study. After a 1-wk acclimation period on basal diet in pellet form (CF-2 Diet; Clea Japan, Tokyo, Japan), 12 animals received drinking water containing BHP (Nakalai Tesque Co. Ltd., Kyoto, Japan) at a concentration of 2000 ppm for 12 wk and then drinking water without BHP. In order to obtain normal lung tissue, the remaining three animals were maintained free from the carcinogen exposure throughout the experimental period. Normal lung tissue was used as control to eliminate contamination with macroscopically undetected cancerous tissue. All rats were killed by exsanguination from the abdominal aorta under light ether anesthesia 25 wk after the beginning of the experiment.

Tissue Preparation

Upon sacrifice, the lungs were immediately excised, and grossly apparent tumors were dissected from their surrounding tissue. Samples were frozen in liquid nitrogen and stored at −80°C until analysis. Portions of the tumors were fixed in 10% neutrally buffered formalin at 4°C and routinely processed for hematoxylin and eosin staining, and histopathologically evaluated according to diagnostic criteria previously described [20,21].

Cloning of 5′-Region of the Rat *Dutt1/Robo1* cDNA

The first 170 nucleotides of the human *ROBO1* gene are not present in the human *DUTT1* gene sequence [10]. Therefore, to identify the 5′-region of the rat *Dutt1/Robo1* cDNA, primers were designated from the mouse Dutt1 cDNA (GenBank: accession number Y17793) and the rat Robo1 cDNA (GenBank: accession number AF041082).

Total RNA was extracted from frozen normal lung tissue with an ISOGEN kit (Nippon Gene, Inc., Toyama, Japan), and first-strand cDNA were synthesized from 1 μg aliquots with a Ready-To-Go Your-Prime First-Strand Beads (Pharmacia Co. Ltd., Tokyo, Japan). The polymerase chain reaction (PCR) amplification was performed in 10 μL of reaction mixture consisting of 1 μM of primers; forward, 5′-TTGTGTAGGGTGTATGGTGT-3′ (from the mouse Dutt1 cDNA), and reverse, 5′-TTGTCTGAAGTCATCCCGTA-3′ (from the rat Robo1 cDNA), 200 μM of each dNTP, 1× PCR buffer (Perkin Elmer, Applied Biosystems Division, Foster City, CA), 2.5 U of Ampli Taq (Perkin Elmer), and 0.5 μL of synthesized cDNA mixture. The reaction conditions were as follows: a primary denaturation step for 2 min at 95°C, 32 cycles of 30 s denaturation at 95°C, 30 s annealing at 58°C, and 1 min extension at 72°C, and a final extension for 10 min at 72°C.

The amplified PCR products were subcloned with a TOPO TA cloning kit (Invitrogen Corp., Carlsbad, CA) and sequenced with a BigDye terminator v3.0 cycle sequencing ready reaction kit (Applied Biosystems Japan Ltd., Tokyo, Japan) and an ABI PRISM 310 genetic analyzer (Applied Biosystems Japan Ltd.).

Reverse Transcription (RT)-PCR Amplification for Aberrant Transcripts of the *Dutt1/Robo1* Gene

Total RNA was extracted from frozen tissue of 12 adenocarcinomas, and first-strand cDNAs were synthesized from 2 μg aliquots with a Ready-To-Go Your-Prime First-Strand Beads (Pharmacia Co. Ltd.). To eliminate possible false positives caused by residual genomic DNA, all samples were treated with DNase.

Nested RT-PCR analysis was performed to analyze for aberrant transcription of the *Dutt1/Robo1* gene, with the primers FA, RA, FB, and RB (Table 1) designated according to the resultant sequences obtained from the above described 5′-region cloning. The first round of PCR amplification was performed in 10 μL of reaction mixture consisting of 1 μM of primers FA and RA, 200 μM of each dNTP, 1× LA PCR buffer (TaKaRa SHUZO Co., Ltd., Shiga, Japan), 0.5 U of TaKaRa LA Taq (TaKaRa SHUZO Co., Ltd.), 0.5 U of Platinum Taq Antibody (Invitrogen Corp.), and 0.5 μL of synthesized cDNA mixture. The reaction comprised a primary denaturation step for 2 min at 95°C, 35 cycles of 30 s denaturation at 95°C, 30 s annealing at 60°C, and 5 min extension at 68°C, and a final extension for 10 min at 72°C. The amplified product was diluted 20-fold in TE buffer, and 1 μL of

Table 1. The Primer Sequences Used in This Study

	Primer	Annealing temperature (°C)
FA	5′-ATTTGGAGATCTCCGCTTCT-3′	60
RA	5′-CAAGTACTTGCATTTCTGCCA-3′	
FB	5′-CTTGGCATGAGACTGTATGC-3′	60
RB	5′-AAGTAGGCCTACAGTACGGA-3′	

the dilutant was subjected to second round of PCR amplification with the same conditions, with primers FB and RB (Table 1). The PCR products were then separated on 1% SeaKem GTG agarose gels (BMA, Rockland, ME) containing 0.05 μg/mL ethidium bromide. Each nested RT-PCR assay was repeated at least twice for confirmation with the originally extracted RNA.

DNA Nucleotide Sequencing

Following the RT-PCR analysis, DNA fragments from aberrant splicing bands in the gels were extracted and reamplified, and the obtained PCR products were directly sequenced with a BigDye terminator v3.0 cycle sequencing ready reaction kit (Applied Biosystems Japan Ltd.) and an ABI PRISM 310 genetic analyzer (Applied Biosystems Japan Ltd.). To confirm the results, each PCR product was sequenced with the forward and reverse primers at least twice.

RT-PCR Amplification for the *Dutt1/Robo1* Gene Expression

PCR amplification was carried out in a reaction volume of 20 μL of first-strand cDNA synthesized in the above experiment. Amplification products for the *Dutt1/Robo1* gene comprised a portion of nucleotides (nt) 4429–4873. This region included no deletion sites. The rat glyceraldehydes-3-phosphate dehydrogenase (*Gapdh*) gene was used as an internal control gene [27]. Primer pairs were as follows: for Dutt1/Robo1, 5′-AGCGAGAGCAGACAGATCAT-3′ (sense) and 5′-TCACATTAGCATCGTAAGCC-3′ (antisense) (annealing temperature: 58°C); and for Gapdh, 5′-TTGTGAAGGTCGGTGTGAAC-3′ (sense) and 5′-AGGGGTCGTTGATGGCAACA-3′ (antisense) (annealing temperature: 55°C) [27]. For each gene, multiple cycles of PCR amplification were tested. The cycle at which a sample having the highest expression reached an amplification plateau was determined, and a cycle number smaller than this was adopted for the analysis.

Preparation of a cDNA Probe and Southern Blot Analysis

A 4.852 kb cDNA fragment for the rat *Dutt1/Robo1* gene was obtained by RT-PCR, with primers FA and RA (Table 1). Total RNA was extracted from frozen normal lung tissue and subjected to 35 cycles of 1 min at 95°C for denaturation, 1 min at 60°C for annealing, and 5 min at 72°C for extension. The amplified product was separated on 1% agarose gels containing 0.05 μg/mL ethidium bromide and the DNA fragment extracted from the gels was cloned and confirmed by sequencing, then used as the hybridization probe for Dutt1/Robo1. Coding region (nt −68 to 4784) was covered by this probe.

Genomic DNAs were extracted from frozen tissues of six adenocarcinomas with a DNeasy tissue kit (QIAGEN, Hilden, Germany). After digestion with the restriction enzyme HindIII (TaKaRa SHUZO Co., Ltd.), 10 μg of DNA samples were fractioned by size in 1% SeaKem GTG agarose gels (BMA), blotted onto Hybond-XL membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK), and hybridized with the [α-^{32}P]dCTP radiolabeled Dutt1/Robo1 probe with the Rediprime II random prime labeling system (Amersham Pharmacia Biotech). Blots were washed and then placed in contact with Hyperfilm MP (Amersham Pharmacia Biotech) at −80°C.

RESULTS

A total of 12 lung adenocarcinomas were obtained, one from each rat treated with BHP, for RT-PCR analysis. Among them, six were sized >8 mm in diameter and could thus be employed for additional Southern blot analysis. We detected 81 bp of the 5′-untranslated sequence and 52 bp of the first nucleotide and thus designated the forward primers for the nested RT-PCR analysis (Figure 1). With 12 adenocarcinomas and three normal lung tissues, we reverse transcribed mRNAs and amplified cDNA by PCR.

The nested RT-PCR analysis revealed that, whereas only the normal product band was amplified at 4696 bp in all three normal lung tissues, one additional abnormal-sized band appeared representing aberrant RT-PCR products with four out of 12 adenocarcinomas (33.3% incidence). Representative RT-PCR results for one normal lung tissue and ten adenocarcinomas, and the followed sequencing data are shown in Figure 2. The abnormal-sized bands were seen at four different position of 1306, 1079, 438, and 432 bp. Sequencing analysis revealed that these aberrant fragments were due to absence of the regions nt 929–4318, 605–4221, 89–4346, and 55–4318 (see Table 2). All normal-sized transcripts demonstrated normal sequences (data not shown). RT-PCR analysis indicated that no reduction or loss of expression of the *Dutt1/Robo1* gene was found in the 12 adenocarcinomas (Figure 3).

With the probe including the deletion sites for aberrant transcription, six adenocarcinomas were assessed by Southern blot analysis. No abnormal restriction patterns, such as deletion or genomic rearrangement, could be detected. Representative results are shown in Figure 4.

DISCUSSION

The present investigation of alterations of the *Dutt1/Robo1* gene in rat lung adenocarcinomas induced by BHP demonstrated aberrant transcripts (33.3% incidence) due to deletions, but no other evidence of genomic abnormality. These results resembled those for the *FHIT* gene obtained in our previous study used this model [19], suggesting that the *Dutt1/Robo1* gene may be another target of chemical carcinogens.

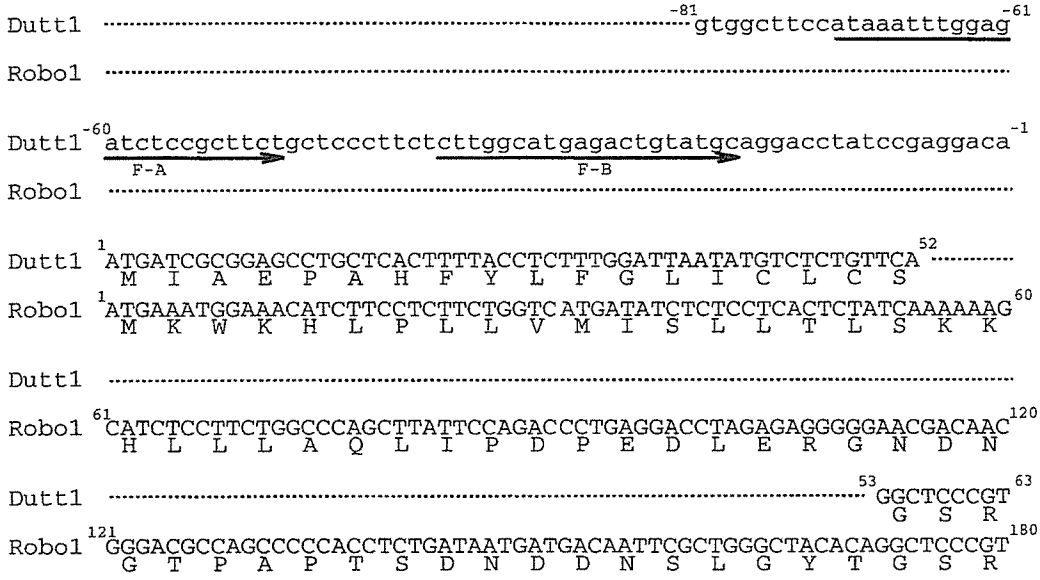

Figure 1. Nucleotide and deduced amino acid sequences of the rat *Dutt1/Robo1* gene, compared with the rat *Roibo1* gene (GenBank: accession number AF041082). Arrows indicate the region used for the forward primers for nested reverse transcription (RT)-polymerase chain reaction (PCR).

The aberrant transcripts detected were due to four patterns of deletions, in exons 2–26, 2–28, 4–26, and 7–26, respectively. Because *DUTT1/ROBO1* gene is composed of 29 exons [10], these deletions would result in loss of the greater part of the *Dutt1/Robo1* gene product. In a recent report, Sundaresen et al. described that they found small-sized RT-PCR products derived from human lung tumor RNAs, including deletion of 327 nucleotides of the *DUTT1/ROBO1* gene [7].

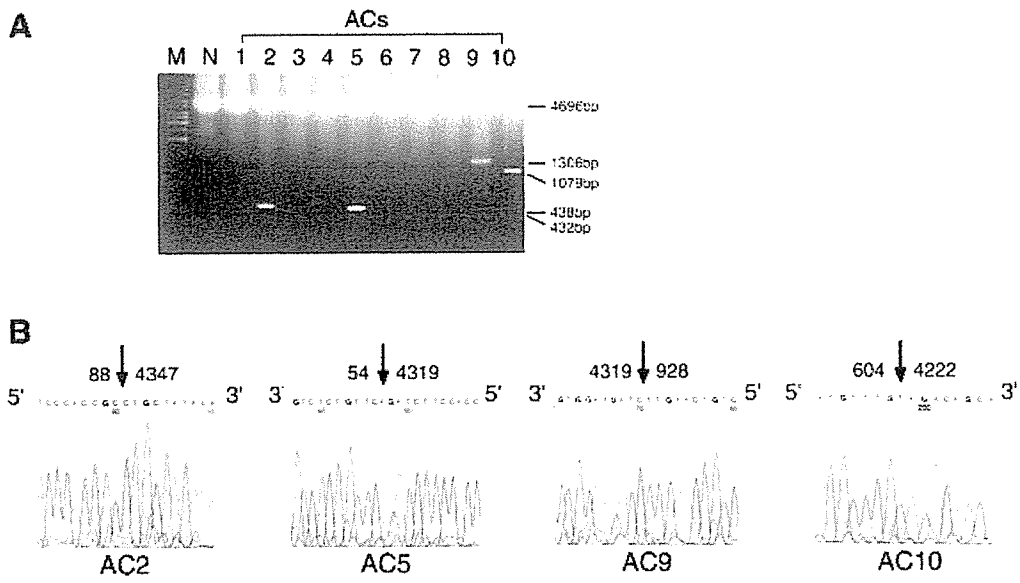

Figure 2. Aberrant transcripts of the *Dutt1/Robo1* gene. (A) Representative results of nested RT-PCR analysis. M, size marker. N, normal lung tissue. ACs, adenocarcinomas. (B) Patterns of aberrant transcripts detected by sequencing analysis. Arrows indicate the junction between nt 88 and 4347 (adenocarcinoma 2), nt 54 and 4319 (adenocarcinoma 5), nt 928 and 4319 (adenocarcinoma 9), and nt 604 and 4222 (adenocarcinoma 10). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Table 2. Summary of the Detected Aberrant Transcripts of the *Dutt1/Robo1* Gene in Rat Lung Adenocarcinomas Induced by *N*-Nitrosobis(2-Hydroxypropyl)Amine (BHP)

Adenocarcinomas	Aberrant transcripts	Four out of 12 adenocarcinomas (33.3% incidence)
2	4258 bp deletion	nt 89–4346
5	4264 bp deletion	nt 55–4318
9	3390 bp deletion	nt 929–4318
10	3617 bp deletion	nt 605–4221

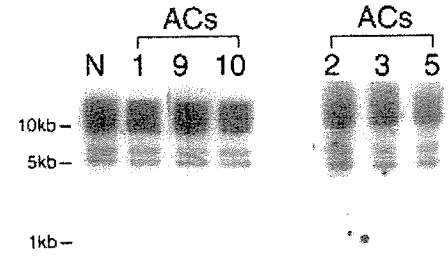

Figure 4. Representative results of the Southern blot analysis of Hind III-digested DNA. N, normal lung tissue; ACs, adenocarcinomas.

Homozygous deletion of the *DUTT1/ROBO1* gene has been found in human cancer cell lines, including one small cell lung cancer (SCLC) cell line [7]. In the present study, however, no homozygous deletion or rearrangement could be detected in any of the adenocarcinomas. It was unclear whether aberrant transcripts were associated with genomic deletions or chromosomal rearrangement in the *DUTT1/ROBO1* gene, although they have been found in the *FHIT* gene in human lung tumor and their derived cell lines [15]. However, in the present model there was no correlation between genomic abnormality and aberrant transcripts in the *Dutt1/Robo1* gene, as in the *FHIT* gene in rat lung adenocarcinomas [19]. Involvement of the loss of heterozygosity and small-sized-deletion of the *Dutt1/Robo1* gene remains to be clarified.

Mutational inactivation of the *DUTT1/ROBO1* gene may be a rare event in the development of tumors. No mutations in any of DUTT1/ROBO1 exons or in the flanking introgeneic sequences were detected in 21 primary invasive breast carcinomas, whereas only two missense mutations with amino acid substitutions were found in 35 SCLC cell lines [10]. Other mechanism of inactivation, such as epigenetic alteration by promoter hypermethylation, is possible with epigenetic silencing of tumor suppressor gene expression in human cancers [28,29]. In fact, one breast tumor cell line lacking expression showed complete hypermethylation of CpG site within the promoter region of the *DUTT1/ROBO1* gene [10]. The same region was found to be hypermethylated in one out of 26 primary NSCLCs (4%)

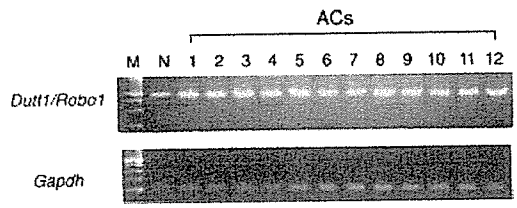

Figure 3. The representative results of the RT-PCR analysis of *Dutt1/Robo1* gene expression. M, size marker; N, normal lung tissue; ACs, adenocarcinomas.

but in none of 23 SCLCs (0%) [10]. In the present study, no reduction or loss of the Dutt1/Robo1 expression could be detected. Therefore, it seems the abnormal expression of the *Dutt1/Robo1* gene, associated with epigenetic inactivation by promoter hypermethylation, may be rare or absent during lung carcinogenesis.

In conclusion, the present study indicated that alteration of the *Dutt1/Robo1* gene may play some limited role in rat lung carcinogenesis induced by BHP, with the gene as a possible target of chemical carcinogens. However, the frequency of alterations was low relative to the FHIT case. To better understand the affects of environmental factors on TSGs located on human chromosome 3p, further investigations appear required.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Health, Labor and Welfare of Japan and by a Grant-in-Aid for Scientific Research Expenses for Health and Welfare Programs, 2nd-Term Comprehensive 10-Year Strategy for Cancer Control, Cancer Prevention, from the Ministry of Health, Labor and Welfare of Japan. The authors express their appreciation to Sachiko Nakai and Megumi Yamaguchi for their technical assistance.

REFERENCES

1. Kok H, Osinga J, Carrit B, et al. Deletion of a DNA sequence at the chromosomal region 3p21 in all major types of lung cancer. Nature (London) 1987;330:578–581.
2. Naylor SL, Johnson BE, Minna JD, Sakaguchi AY. Loss of heterozygosity of chromosome 3p markers in small-cell lung cancer. Nature (London) 1987;329:451–454.
3. Brauch H, Johnson B, Hovis J, et al. Molecular analysis of the short arm of chromosome 3 in small-cell and non-small-cell carcinoma of the lung. N Engl J Med 1987;317:110–1113.
4. Yokota J, Wada M, Shimosato Y, Terada M, Sugimura T. Loss of heterozygosity on chromosome 3, 13, 17 in small cell carcinoma and chromosome 3 in adenocarcinoma of the lung. Proc Natl Acad Sci USA 1987;84:9252–9256.
5. Kok K, Naylor SL, Buys CH. Deletions of the short arm of chromosome 3 in solid tumors and the search for suppressor genes. Adv Cancer Res 1997;71:27–92.