Mutations and Reduced Expression of the Transforming Growth Factor-β Receptor II Gene in Rat Lung Adenocarcinomas Induced by N-Nitrosobis(2-hydroxypropyl)amine

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Mutations and expression of the transforming growth factor-β receptor type II (TGF-βRII) gene were investigated in lung adenocarcinomas induced by N-nitrosobis(2-hydroxypropyl)amine (BHP) in rats. Males of the Wistar strain, 6 weeks old, were given 2000 ppm of BHP in their drinking water for 12 weeks and then maintained without further treatment until killed at week 25. Total RNA was extracted from 12 adenocarcinomas and mutations in TGF-βRII were investigated by RT-PCR-restriction-SSCP analysis followed by sequencing analysis. Two out of 12 adenocarcinomas showed band shifts, indicative of mutations (16.7%). One was a CTG-to-TTG (Leu to Leu) transition at codon 308 without amino acid alteration and the other a frameshift deletion of one of two guanines at nucleotides 1434 to 1435 (codon 477 to 478). Semi-quantitative RT-PCR analysis demonstrated significantly reduced TGF-βRII expression in adenocarcinomas, as compared with normal lung tissue. These results suggest that TGF-βRII alterations may play a role in the acquisition of growth advantage by lung adenocarcinomas induced by BHP in rats.

Key words: TGF-βRII — Lung adenocarcinoma — Nitrosamine — Rat

Transforming growth factor-β (TGF-β) is a multifunctional polypeptide, which regulates a wide variety of cell functions, including division, differentiation, apoptosis, migration and adhesion,1–4 primarily by binding to specific cell surface proteins, TGF-β receptors. The major types of these latter are referred to as TGF-β receptor type I (TGF-βRI), TGF-β receptor type II (TGF-βRII) and TGF-β receptor type III (TGF-βRIII). TGF-β signals may pass through a heteromeric complex of TGF-βRI and TGF-βRII, both of which belong to an emerging family of transmembrane serine/threonine kinases.6,7 TGF-β binds directly to TGF-βRII, whereas TGF-βRI appears to recognize only TGF-β that is bound to TGF-βRII, with resultant recruitment into a ternary signaling complex. TGF-βRII then phosphorylates TGF-βRII, which propagates further downstream signals.7–9 Since TGF-β is a potent negative regulator of cell proliferation,10,11 it is likely that alterations of TGF-β receptors contribute to uncontrolled proliferation of cells in vivo. Therefore, it has been suggested that the gene encoding TGF-βRII may act as a tumor suppressor gene.

Recently, alterations of TGF-βRII have been detected in several cancers.12–18 In human small cell lung carcinomas (SCLCs), mutations are infrequent but expression may be reduced, as has been documented for non-small cell lung carcinomas (NSCLCs).19–22 In rodents, reduced expression of TGF-βRII has also been reported in mouse lung tumors.23 However, to our knowledge, no investigation of TGF-βRII genetic alteration in rat lung tumors has hitherto been performed. Previously, we described a model for the development of NSCLCs in rats given N-nitrosobis(2-hydroxpropyl)amine (BHP) in drinking water, with high yields of adenomatous lesions, including adenocarcinomas,24–26 which is useful for investigation of molecular mechanisms. Recently, we found mutations of Smad2 and Smad4 genes in the resultant lung adenocarcinomas.27 It has been demonstrated that members of the Smad family of proteins play important roles in TGF-β-mediated signal transduction.28,29 Therefore, in the present experiment, we investigated mutations and expression of TGF-βRII in adenocarcinomas induced by BHP to clarify the involvement of the TGF-β signaling pathway in their development.

Male Wistar rats, 5 weeks old, were purchased from Japan SLC Inc. (Shizuoka) and housed 3–5 to a plastic cage 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-week acclimation period on a basal diet in pellet form (Oriental MF Diet; Oriental Yeast Co., Ltd., Tokyo), the animals received 2000 ppm of BHP (Nacalai Tesque Co., Ltd., Kyoto) in their drinking water for 12 weeks and then drinking water without supplement. They were killed.
under ether anesthesia 25 weeks after the beginning of the experiment. At sacrifice, the lungs were immediately excised and grossly apparent tumors were dissected from surrounding tissue. Samples were frozen in liquid nitrogen, and stored at −80°C until analysis. Portions of the tumors were also fixed in 10% formalin for routine processing and staining with hematoxylin and eosin (H&E) for histological examination.

Total RNA was extracted from frozen tissue using ISOGEN (Nippon Gene, Inc., Toyama) and first-strand cDNA was synthesized from 5 µg samples with Ready-To-Go Your-Prime First-Strand Beads (Pharmacia Co. Ltd., Tokyo). To eliminate possible false positives caused by residual genomic DNA, all samples were treated with DNase. RT-PCR-restriction-SSCP analysis was carried out using the primers listed in Table I. All were designed from rat TGF-βRII cDNA sequences (GenBank: accession number L09653). PCR amplification was performed with 10 µl reaction mixtures consisting of 1 µM of each primer, 200 µM of each dNTP, 1× PCR buffer (Perkin Elmer, Applied Biosystems Division, Foster City, CA), 68 nM [α-32P]-dCTP, 2.5 units 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 62°C or 64°C and 2 min at 72°C, and a final extension for 10 min at 72°C. To rule out PCR artifacts, PCR amplification was repeated from individual original DNA at least once. Amplified fragments were digested to shorter than 300 bp with restriction enzyme before electrophoresis,27) as indicated in Table I. PCR products were diluted with 90 µl of loading solution containing 90% formamide, 20 mM EDTA, and 0.05% xylene cyanol and bromophenol blue, denatured at 90°C for 2 min and applied to 6 or 10% polyacrylamide gels containing 10% 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. DNA fragments of mobility-shifted bands identified by SSCP analysis were extracted from the gels and reamplified. The PCR products obtained were cloned using a TOPO TA cloning kit (Invitrogen Corporation, Carlsbad, CA) and recombinant plasmid DNA clones were sequenced with Sequencing Pro (Toyobo Co., Ltd., Tokyo). In each experiment, 5 to 10 clones from different bacterial colonies were investigated.

Semi-quantitative RT-PCR analysis was performed as described previously.30) PCR amplification was carried out in a reaction volume of 20 µl containing 1 µl of first-strand cDNA synthesized in the above experiment. Amplification products comprising a portion of 333 to 1352 nucleotides for TGF-βRII (primer: 2-F and 3-R), and exons 5 through 8 of the glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH)31) were generated from the cDNA template in parallel PCRs. The PCR conditions were as follows; a denaturation step for 5 min at 95°C, 26 cycles of 1 min at 95°C, 1 min at 67°C, and 2 min at 72°C, with a final extension step for 10 min at 72°C, using both the TGF-βRII and GAPDH primers. PCR products were then separated on 2% agarose gels containing 0.05 µg/ml ethidium bromide. Each RT-PCR assay was repeated at least twice for confirmation. To quantify relative levels of TGF-βRII expression, the electrophoresed PCR products were visualized under UV light and photographed. The bands were quantitated with image analysis software (NIH Image, Bethesda, MD) and peak intensities for PCR products derived for TGF-βRII were divided by those for GAPDH. Quantitative differences between adenocarcinomas and normal lung tissues were analyzed for statistical significance using Student’s t test.

A total of 12 adenocarcinomas induced by BHP in 12 rats were used for the analysis. Three normal lung tissues of untreated rats were used as controls, because of the pos-

| cDNA location | Primer | Size of amplified product (bp) | Annealing temperature (°C) | Enzyme digestion | Size of digested product (bp) |
|---------------|--------|-------------------------------|---------------------------|------------------|-----------------------------|
| nt 33–413     | 1-F : 5′-GCTGCACATCGTCTGTTGGA-3′ 1-R : 5′-CAGGACGACATGAAGAGGT-3′ | 381                       | 62                         | PstI             | 182, 199                     |
|               | 2-F : 5′-CCTCTGAAAGATGCCCATT-3′ 2-R : 5′-TCCACGAGGTATTCTCG-3′ | 530                       | 62                         | BclI             | 286, 244                     |
| nt 333–862    | 3-F : 5′-GAGACCGCTGGCAGTCAAGA-3′ 3-R : 5′-GCCATGGTAGACATCCGT-3′ | 540                       | 64                         | SmaI             | 254, 286                     |
| nt 813–1352   | 4-F : 5′-GGGAACACCGGATACATG-3′ 4-R : 5′-GGAGTCTTGGGCCAGCCTGC-3′ | 479                       | 64                         | MboI             | 277, 202                     |

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sibility that non-cancerous portions of BHP-treated rats may include small microscopic lesions which are undetectable macroscopically, such as not only adenocarcinomas, but also hyperplasias and adenomas. The results of PCR-restriction-SSCP analysis and sequencing analysis of TGF-βRII gene mutations are shown in Fig. 1, (A) and (B). Two out of 12 adenocarcinomas showed band shifts in the regions of nt 813–1066 and nt 1256–1532, indicative of mutations (16.7%). These were established to be a CTG-to-TTG (Leu to Leu) transition at codon 308, and sample 4 a frameshift deletion of one of two guanines at nucleotides 1434 to 1435 (codon 477 to 478). The latter would be expected to result in truncation of the TGF-βRII protein, 81 amino acids downstream.

Representative results of semi-quantitative RT-PCR analysis for TGF-βRII expression are shown in Fig. 2 and the results of quantification by densitometry, normalized with respect to the GAPDH value, are summarized in Table II. The levels of TGF-βRII expression in adenocarcinomas were significantly lower than those of normal lung tissues ($P < 0.01$). Reduction in 9 out of 12 adenocarcinomas was to approximately 0.09 to 0.42 fold, as compared with normal lung tissue.

Recently, mutations of the TGF-βRII gene have been reported in several human cancers. Especially high frequencies were detected in colorectal cancers and cell lines, and gastric cancer cell lines with microsatellite instability (MSI).12–16) In these tumors, TGF-βRII is mutated primarily by deletion or insertion of one or two additional adenine base pairs within a 10-base-pair polyadenine tract (codons 125–128) in the coding region, suggesting a close association with MSI.12–16) These frameshift mutations result in truncation of proteins that lack the transmembrane and intracellular domains of TGF-βRII and consequently are inactive for downstream signaling,12) leading cancer cells to become resistant to TGF-β. However, TGF-βRII mutations have also been detected in gastric cancer without microsatellite instability.15) Furthermore, inactivating mutations in the serine/threonine kinase domain of TGF-βRII have been reported in head and neck squamous carcinomas.17) In SCLCs with a replication error phenotype, TGF-βRII mutation is infrequent in the polyadenine tract, and it is absent in NSCLCs, indicating rare occurrence in lung cancers.19)

It is considered that the G/C-to-A/T transition is a common mutation pattern induced by ethylating N-nitroso compounds.32) Previously, we have reported Ki-ras mutations to all be G/C-to-A/T transitions at codon 12 in pre-neoplastic and neoplastic lung lesions induced by BHP in rats.26) Therefore, the C/G-to-T/A transition at codon 308
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Table II.  Results of Semi-quantitative Densitometric RT-PCR Analysis of TGF-βRII Expression and the Mutational Analysis of TGF-βRII, Smad2 and Smad4 Genes in Lung Adenocarcinomas Induced by BHP in Rats

| Samples       | TGF-βRII expression | Mutations |
|---------------|---------------------|-----------|
|               |                     | TGF-βRII  | Smad2 | Smad4 |
| Normal lung tissues | 1.089               | WT | WT | WT |
| 2             | 1.325               | WT | WT | WT |
| 3             | 1.254               | WT | WT | WT |
| mean±SD      | 1.22±0.12           |         |       |       |
| Adenocarcinomas | 0.438               | WT | WT | WT |
| 2             | 0.344               | WT | WT | WT |
| 3             | 0.296               | WT | WT | WT |
| 4             | 0.276               | Mut | Mut | WT |
| 5             | 1.228               | WT | WT | WT |
| 6             | 0.473               | WT | WT | WT |
| 7             | 1.054               | WT | WT | WT |
| 8             | 0.227               | WT | Mut | WT |
| 9             | 0.367               | WT | WT | Mut |
| 10            | 1.097               | WT | WT | WT |
| 11            | 0.110               | WT | WT | WT |
| 12            | 0.516               | Mut | WT | WT |
| mean±SD      | 0.54±0.38           |         |       |       |

a) Normalized to GAPDH values.
b) Significantly lower than normal lung tissue level (P<0.01).
c) Published in Ref. 27.
WT, wild; Mut, mutated.

in TGF-βRII may have been caused by BHP. However, it is not clear why one of the two guanines at nucleotides 1434 to 1435 was deleted.

In RT-PCR-restriction-SSCP analysis, since the adenocarcinoma #4 seems to retain its wild-type allele (Fig. 1(A)), it is possible that TGF-βRII function was not inactivated. However, this case showed reduced expression of TGF-βRII (Fig. 2, Table II). Loss or reduction of TGF-βRII expression has also been related to resistance to TGF-β inhibition of cell proliferation. In a number of neoplasms, including gastric cancer cell lines, thyroid tumors, SCLCs and NSCLCs, reduced expression or failure to express the TGF-βRII gene at the RNA or protein level has been observed without apparent structural mutation. In rodents, reduced expression of TGF-βRII has been reported in mouse lung tumors induced by urethane, with a possible contribution to autonomous cell growth. In the present study, the fact of reduced expression of TGF-βRII in 9 out of 12 adenocarcinomas (75.0%), including adenocarcinoma #4, indicates that this may play an important role, rather than mutation, since changes occurring down stream of the signaling pathway would affect the transactivation of target genes. Further studies are needed to solve the molecular mechanisms of lung cancer development induced by nitrosamines.

In conclusion, taken together with our recent results on Smad2 and Smad4 mutations, the present findings suggest a possible involvement of alterations in the TGF-β signaling pathway in the development of lung adenocarcinomas induced by BHP in rats.

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