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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-β RI), TGF-β receptor type II (TGF-βRII) and TGF-β receptor type III (TGF-βRIII).[5] 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-βRI, 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-hydroxypropyl)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

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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 μ*M* of each primer, 200 μ*M* of each dNTP, 1× PCR buffer (Perkin Elmer, Applied Biosystems Division, Foster City, CA), 68 n*M* [α-^{32}P] 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 m*M* 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 0.5X Tris-borate EDTA buffer with or without 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-

Table I. The Primer Sequences of TGF-βRII Used in This Study

cDNA location	Primer	Size of amplified product (bp)	Annealing temperature (°C)	Enzyme digestion	Size of digested product (bp)
nt 33−413	1-F : 5′-GCTGCACATCGTCCTGTGGA-3′ 1-R : 5′-CAGGAGCACATGAAGAAGGT-3′	381	62	PstI	182, 199
nt 333−862	2-F : 5′-CACTCTGGAAGATGCCACTT-3′ 2-R : 5′-TCCACGAGGAGTATTCCTCG-3′	530	62	BcnI	286, 244
nt 813−1352	3-F : 5′-TGAGACCGTGGCTGTCAAGA-3′ 3-R : 5′-GCCATGGAGTAGACATCCGT-3′	540	64	SmaI	254, 286
nt 1256−1734	4-F : 5′-TGGGAACAGCGAGATACATG-3′ 4-R : 5′-GGAGTCTTGGCCCAGCCTGC-3′	479	64	MboI	277, 202

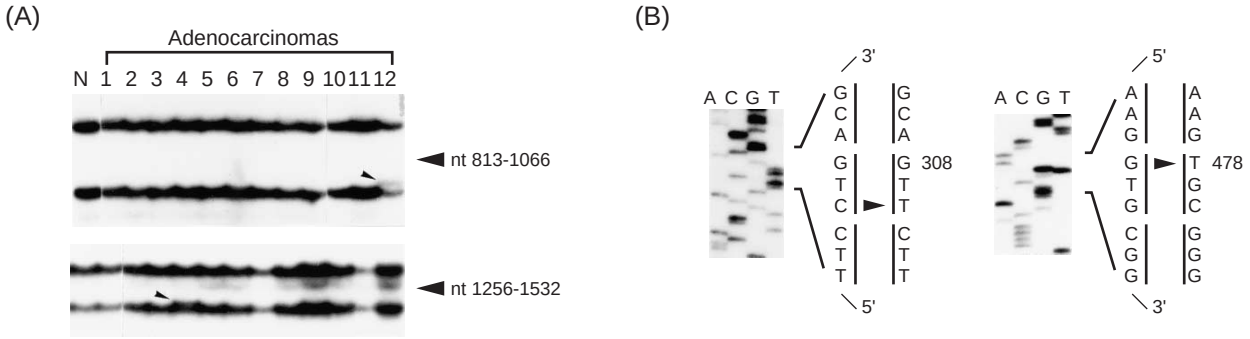

Fig. 1. (A) Representative results of RT-PCR-restriction-SSCP analysis for TGFβRII mutations in lung adenocarcinomas induced by BHP in rats. Samples 12 and 4 exhibit band shifts in the regions of nt 813–1066 and nt 1256–1532 of TGFβRII (arrowheads), respectively. N: normal lung tissue. (B) Results of sequencing analysis of the TGFβRII mutations in lung adenocarcinomas induced by BHP in rats. Sample 12 showed 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.

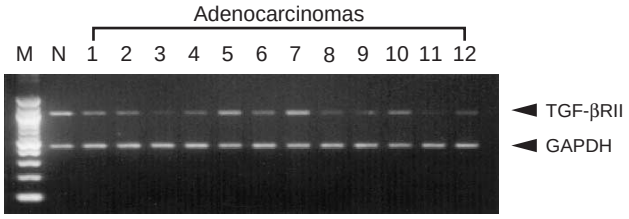

Fig. 2. Representative results of semi-quantitative RT-PCR analysis of TGFβRII expression in lung adenocarcinomas induced by BHP in rats. Amplification was performed in a 20-μl volume, using both TGFβRII and GAPDH primers, and the products were separated by 2% agarose gel electrophoresis. M, 100 bp DNA size marker; N, normal lung tissue.

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 two regions of nucleotides (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 a frameshift deletion of one of two guanines at nt 1434 to 1435 (codons 477 to 478), respectively. The former results in no amino acid alteration and the latter was expected to result in truncation of the TGF-βRII protein 81 amino acids downstream. Both mutations were located in the serine/threonine kinase domain.

Representative results of semi-quanititative 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 preneoplastic and neoplastic lung lesions induced by BHP in rats.[26] Therefore, the C/G-to-T/A transition at codon 308

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[a)]	Mutations		
			TGF-βRII	Smad2[c)]	Smad4[c)]
Normal lung tissues	1	1.089	WT	WT	WT
	2	1.325	WT	WT	WT
	3	1.254	WT	WT	WT
	mean±SD	1.22±0.12			
Adenocarcinomas	1	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[b)]			

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.[15, 18, 20–22)] In a number of neoplasms, including gastric cancer cell lines,[15)] thyroid tumors,[18)] SCLCs[20, 21)] and NSCLCs,[22)] 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.[23)] In the present study, the fact of reduced expression of TGF-βRII in 9 out of 12 adenocarcarcinomas (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,[27)] 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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REFERENCES

1) Rizzino, A. Transforming growth factor-β: multiple effects on cell differentiation and extracellular matrices. *Dev. Biol.*, **130**, 411–422 (1988).
2) Roberts, A. B. and Sporn, M. B. The transforming growth factor-betas. *In* "Peptide Growth Factors and Their Receptors—Handbook of Experimental Pharmacology," ed. M. B. Sporn and A. B. Roberts, pp. 419–472 (1990). Springer-Verlag, Heidelberg.
3) Roberts, A. B. and Sporn, M. B. Physiological actions and clinical applications of transforming growth factor-beta (TGF-beta). *Growth Factors*, **8**, 1–9 (1993).
4) Kingsley, D. M. The TGF-β superfamily: new members, new receptors, and genetic tests of function in different organisms. *Genes Dev.*, **8**, 133–146 (1994).
5) Massague, J., Attisano, L. and Wrana, J. L. The TGF-β family and its composite receptors. *Trends Cell Biol.*, **4**, 172–178 (1994).
6) Lin, H. Y., Wang, X. F., Ng-Eaton, E., Weinberg, R. A. and Lodish, H. F. Expression cloning of the TGF-β type II receptor, a functional transmembrane serine/threonine kinase. *Cell*, **68**, 775–785 (1992).
7) Wrana J. L., Attisano, L., Wieser, R., Ventura, F. and Massague, J. Mechanism of activation of the TGF-β receptor. *Nature*, **370**, 341–347 (1994).
8) Franzen, P., ten Dijke, P., Ichijo, H., Yamashita, H., Schulz, P., Heldin, C. H. and Miyazono, K. Cloning of the TGF-β type I receptor that forms a heteromeric complex with the TGF-β type II receptor. *Cell*, **75**, 681–692 (1993).
9) Wieser, R., Wrana, J. L. and Massague, J. GS domain mutations that constitutively activate TβR-I, the downstream signaling component in the TGF-β receptor complex. *EMBO J.*, **14**, 2199–2208 (1995).
10) Sun, L., Wu, G., Willson, J. K., Zborowska, E., Yang, J., Rajkarunanayake, I., Wang, J., Gentry, L. E., Wang, X. F. and Brattain, M. G. Expression of transforming growth factor beta type II receptor leads to reduced malignancy in human breast cancer MCF-7 cells. *J. Biol. Chem.*, **269**, 26449–26455 (1994).
11) Wang, J., Sun, L., Myeroff, L., Wang, X., Gentry, L. E., Yang, J., Liang, J., Zborowska, E., Markowitz, S. and Willson, J. K. Demonstration that mutation of the type II transforming growth factor beta receptor inactivates its tumor suppressor activity in replication error-positive colon carcinoma cells. *J. Biol. Chem.*, **270**, 22044–22049 (1995).
12) Markowitz, S., Wang, J., Myeroff, L., Parsons, R., Sun, L., Lutterbaugh, J., Fan, R. S., Zborowska, E., Kinzler, K. W., Vogelstein, B., Brattain, M. and Willson, J. K. V. Inactivation of the type II TGF-β receptors in colon cancer cells with microsatellite instability. *Science*, **268**, 1336–1338 (1995).
13) Lu, S. L., Akiyama, Y., Nagasaki, H., Saito, K. and Yuasa, Y. Mutations of the transforming growth factor-beta type II receptor gene and genomic instability in hereditary nonpolyposis colorectal cancer. *Biochem. Biophys. Res. Commun.*, **216**, 452–457 (1995).
14) Parsons, R., Myeroff, L. L., Liu, B., Willson, J. K. V., Markowitz, S. D., Kinzler, K. W. and Vogelstein, B. Microsatellite instability and mutations of the tranforming growth factor β type II receptor gene in colorectal cancer. *Cancer Res.*, **55**, 5548–5550 (1995).
15) Park, K., Kim, S. J., Bang, Y. J., Park, J. G., Kim, N. K., Roberts, A. B. and Sporn, M. B. Genetic changes in the transforming growth factor beta (TGF-beta) type II receptor gene in human gastric cancer cells: correlation with sensitivity to growth inhibition by TGF beta. *Proc. Natl. Acad. Sci. USA*, **91**, 8772–8776 (1994).
16) Myeroff, L. L., Parsons, R., Kim, S.-J., Hedrick, L., Cho, K. R., Orth, K., Mathis, M., Kinzler, K. W., Lutterbaugh, J., Park, K., Bang, Y.-J., Lee, H. Y., Park, J.-G., Lynch, H. T., Roberts, A. B., Vogelstein, B. and Markowitz, S. D. A transforming growth factor β receptor type II gene mutation common in colon and gastric but rare in endometrial cancers with microsatellite instability. *Cancer Res.*, **55**, 5545–5547 (1995).
17) Garrigue-Antar, L., Munoz-Antonia, T., Antonia, S., Gesmonde, J., Vellucci, V. F. and Reiss, M. Missense mutations of the transforming growth factor β type II receptor in human head and neck squamous carcinoma cells. *Cancer Res.*, **55**, 3982–3987 (1995).
18) Lazzereschi, D., Ranieri, A., Mincione, G., Taccogna, S., Nardi, F. and Colletta, G. Human malignant thyroid tumors displayed reduced levels of transforming growth factor β receptor type II messenger RNA and protein. *Cancer Res.*, **57**, 2071–2076 (1997).
19) Tani, M., Takenoshita, S., Kohno, T., Hagiwara, K., Nagamachi, Y., Harris, C. C. and Yokota, J. Infrequent mutations of the transforming growth factor beta-type II receptor gene at chromosome 3p22 in human lung cancers with chromosome 3p deletions. *Carcinogenesis*, **18**, 1119–1121 (1997).
20) de Jonge, R. R., Garrigue-Antar, L., Vellucci, V. F. and Reiss, M. Frequent inactivation of the transforming growth factor β type II receptor in small-cell lung carcinoma cells. *Oncol. Res.*, **9**, 89–98 (1997).
21) Norgaard, P., Spang-Thomsen, M. and Poulsen, H. S. Expression and autoregulation of transforming growth factor β receptor RNA in small-cell lung cancer cell lines. *Br. J. Cancer*, **73**, 1037–1043 (1996).
22) Kim, W. S., Park, C., Jung, Y.-S., Kim, H.-S., Han, J., Park, C. H., Kim, K., Kim, J., Shim, Y. M. and Park, K. Reduced transforming growth factor-β type II receptor (TGF-β RII) expression in adenocarcinoma of the lung. *Anticancer Res.*, **19**, 301–306 (1999).
23) Jakowlew, S. B., Moody, T. W., You, L. and Mariano, J. M. Reduction in transforming growth factor-β type II receptor in mouse lung carcinogenesis. *Mol. Carcinog.*, **22**, 46–56 (1998).
24) Konishi, Y., Denda, A., Kondo, H. and Takahashi, S. Lung

carcinomas induced by oral administration of N-bis(2-hydroxypropyl)nitrosamine in rats. *Gann*, **67**, 773–780 (1976).
25) Konishi, Y., Kondoh, H., Denda, A., Takahashi, S. and Inui, S. Lung carcinomas induced by oral administration of N-bis(2-hydroxypropyl)nitrosoamine in rats. *In* "Tumors of Early Life in Man and Animals, Quadrennial International Conference of Cancer. Division of Cancer Research," ed. I. Severi, pp. 637–649 (1978). Perugia University, Perugia.
26) Kitada, H., Tsutsumi, M., Tsujiiuchi, T., Takahama, M., Fukuda, T., Narita, N. and Konishi, Y. Frequent mutations of Ki-ras but no mutations of Ha-ras and p53 in lung lesions induced by N-nitrosobis(2-hydroxypropyl)amine in rats. *Mol. Carcinog.*, **15**, 276–283 (1996).
27) Tsujiuchi, T. Sasaki, Y., Tsutsumi, M. and Konishi, Y. Mutations of Smad2 and Smad4 genes in lung adenocarcinomas induced by N-nitrosobis(2-hydroxypropyl)amine in rats, *Mol. Carcinog.* (2000) in press.
28) Liu, F., Hata, A., Baker, J., Doody, J., Carcamo, J., Harland, R. and Massague, J. A human Mad protein acting as a BMP-regulated transcriptional activator. *Nature*, **381**, 620–623 (1996).
29) Yingling, J., Das, P., Savage, S., Zhang, M., Padgett, R. and Wang, X. Mammalian dwarfins are phosphorylated in response to transforming growth factor β and are implicated in control of cell growth. *Proc. Natl. Acad. Sci. USA*, **93**, 8940–8944 (1996).
30) Tsujiuchi, T., Tsutsumi, M., Sasaki, Y., Takahama, M. and Konishi, Y. Hypomethylation of CpG sites and c-*myc* gene overexpression in hepatocellular carcinomas, but not hyperplastic nodules, induced by a choline-deficient L-amino acid-defined diet in rats. *Jpn. J. Cancer Res.*, **90**, 909–913 (1999).
31) Swafford, D. S., Middleton, S. K., Palmisano, W. A., Nikula, K. J., Tesfaigzi, J., Baylin, S. B., Herman, J. G. and Belinsky, S. A. Frequent aberrant methylation of p16INK-4a in primary rat lung tumors. *Mol. Cell. Biol.*, **17**, 1366–1374 (1997).
32) Jiao, J., Pienkowska, M., Glickman, B. W. and Zielenska, M. Molecular analysis of mutations induced by ethylating *N*-nitroso compounds in the *lac I* gene of *Eschericha coli*. *Mutat. Res.*, **352**, 39–45 (1996).