RAPID COMMUNICATION Jpn. J. Cancer Res. 88, 340–343, April 1997

# *MAD*-related Genes on 18q21.1, *Smad2* and *Smad4*, Are Altered Infrequently in Esophageal Squamous Cell Carcinoma

Chihaya Maesawa,[1,3] Gen Tamura,[1] Satoshi Nishizuka,[1] Takeshi Iwaya,[1] Satoshi Ogasawara,[2] Kaoru Ishida,[2] Ken Sakata,[1] Nobuhiro Sato,[2] Kenichiro Ikeda,[2] Yusuke Kimura,[2] Kazuyoshi Saito[2] and Ryoichi Satodate[1]

*Departments of [1]Pathology and [2]Surgery, Iwate Medical University School of Medicine, 19-1 Uchimaru, Morioka 020*

**The *MAD* (mothers against decapentaplegic)-related genes, *Smad2* (former name *MADR2* or *JV18-1*) and *Smad4* (former name *DPC4*), have been identified on chromosome 18q21.1. We analyzed 30 primary esophageal squamous cell carcinomas (ESCC) and 7 cell lines derived from ESCC for intragenic mutations and loss of heterozygosity (LOH) of the *Smad2* and *Smad4* genes. LOH was detected in 5 of 14 (35%) informative cases. However, no mutations in either gene were detected in either the primary carcinomas or the cell lines, and only a G-to-A base transition within the 3′-untranslated region of the *Smad4* gene was observed in a carcinoma. There were no homozygous deletions in either of the genes in the cell lines. *MAD*-related genes on chromosome 18q21.1 are altered infrequently in ESCC.**

Key words: *Smad2* — *Smad4* — Mutation — Esophageal carcinoma — TGFβ

Transforming growth factor β (TGFβ) is a potent antiproliferative factor for a broad range of epithelial cells.[1,2] The *MAD* (mothers against decapentaplegic) gene from *Drosophila melanogaster*[3,4] and the related *Sma*[5] and *Xmad*[6] genes from *Caenorhabditis elegans* and *Xenopus*, respectively, are essential components of the TGFβ superfamily (TGFβ/activin/bone morphoproteins)-induced signaling pathways. Recently, several human homologous genes encoding MAD-related proteins, including *DPC4*,[7] *MADR1/Samd1*,[8,9] and *MADR2/JV18-1*,[10,11] have been identified. The *DPC4* and *MADR2/JV18-1* genes are located in close proximity at 18q21.1.[7,11] Recently, the nomenclature of these *MAD*-related genes in the vertebrates has been unified by Derynck *et al.* (*MADR2* and *DPC4* are termed *Smad2* and *Smad4*, respectively).[12] Homozygous deletion and intragenic mutations of the *Smad4* gene have been detected in about 30% and 20% of pancreatic carcinomas, respectively.[7,13] Mutations in the *Smad2* gene, accompanied with a loss of heterozygosity (LOH),[10,11] were also detected in colorectal carcinomas. Biochemical analyses have indicated that mutations in the *Smad2* gene lead to decreased protein expression or a loss of TGFβ-regulated phosphorylation.[11] These results suggest that *Smad2* gene is a tumor suppressor gene. Indeed, Eppert *et al.*[11] have suggested that both genes could be implicated as tumor suppressor genes in a variety of gastrointestinal tract tumors.

We assessed 30 primary esophageal squamous cell carcinomas (ESCC) and 7 cell lines derived from ESCC for intragenic mutations in the *Smad2* and *Smad4* genes and LOH at 18q21.1, by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP), reverse transcriptase (RT)-PCR-SSCP, and PCR-microsatellite analyses.

After informed consent had been obtained, thirty primary tumors and matching normal tissues were collected from patients with ESCC. All the cell lines (TE-2, -3, -5, -10, -11, -12, and -13) used in this study were derived from ESCC and were kindly provided by Dr. Testuro Nishihira (Department of Surgery, Tohoku University, Miyagi). Genomic DNA from these materials was extracted by proteinase K digestion and phenol/chloroform extraction. RNA was isolated using Trizol reagent (Gibco, BRL, Gaithersburg, MD).

Intragenic mutations in the *Smad4* gene were screened by PCR-SSCP analysis. The amino (exons 1–3) and carboxyl (exons 8–11) termini of the *Smad4* gene are highly conserved among members of the MAD family, and all identified mutations have been localized in these regions.[7,12,14] Primer sequences used for amplification of these exons have been described.[14] One hundred nanograms of genomic DNA was amplified in a total reaction volume of 20 μl containing 20 pmol of each primer, 1 m*M* $MgCl_2$, and 1 μl of [α-$^{32}$P]dCTP (3000 Ci/mmol, 10 Ci/ml). PCR conditions were as follows: 35 cycles at 95°C for 1 min, the appropriate annealing temperature for 1 min, and 72°C for 1 min. The annealing temperature for exons 1, 2, 3, 8, and 9 was 58°C, and that for exons 10 and 11 was 55°C. Five microliters of each PCR product was diluted 10-fold with gel-loading buffer (98%

[3] To whom correspondence should be addressed.

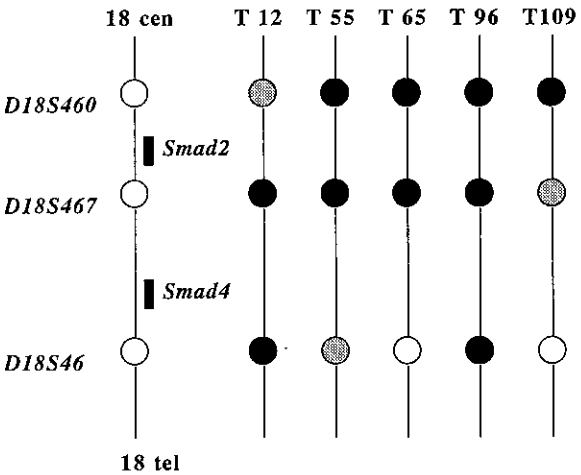


Fig. 1. Deletion map at 18q21.1 in 5 ESCCs. ● LOH, ○ heterozygosity, ◎ homozygosity.

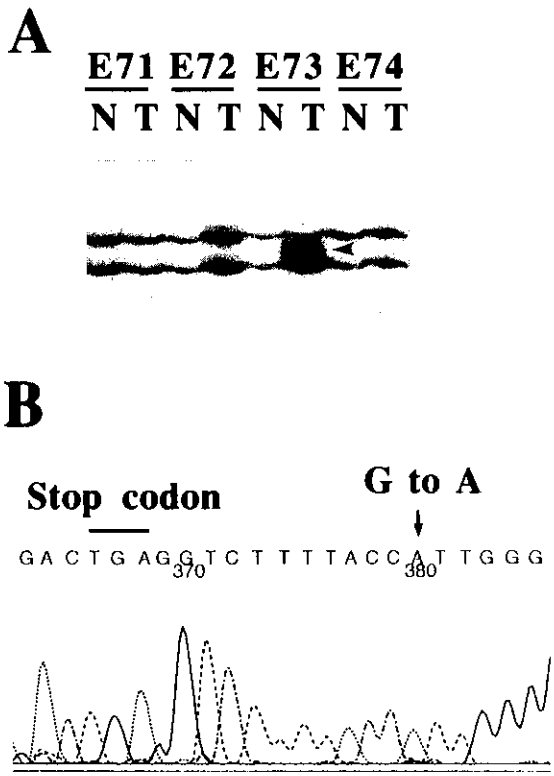


Fig. 2. SSCP analysis (A) and sequencing histogram (B) of exon 11 of the *DPC4* gene. One shifted band is indicated by an arrowhead in specimen E73. A base transition of G to A, nucleotide number 1799 as defined by Hahn *et al.*,[7] located within the 3′-untranslated region of the *Smad4* gene, is found in E73 (arrow).

deionized formamide, 10 m*M* EDTA (pH 8.0), 0.025% xylene cyanol, 0.025% bromophenol blue) and heated at 94°C for 2 min. The samples were electrophoresed on a 6% neutral polyacrylamide gel, with or without 10% glycerol, at 25 to 40 W for 3 to 10 h, at 4°C or room temperature.

RT-PCR-SSCP analysis was performed on the MH1 (N-terminus) and MH2 (C-terminus) domains of *Smad2*, as previously described.[11] Five hundred nanograms of total RNA was reverse-transcribed using random hexamers and AMV (TaKaRa, Tokyo). One hundred nanograms of cDNA was amplified using primers dividing MH1 and MH2 into four regions according to Eppert *et al.*[11] PCR conditions were essentially as described for the *Smad4* gene. All regions were annealed at 55°C. Samples were electrophoresed on a 6% neutral polyacrylamide gel, with or without 10% glycerol, at 5 to 25 watt-hour for 4 to 8 h, at 4°C or room temperature.

Three microsatellite markers on 18q21.1, *D18S46, D18S467, D18S460* (Research Genetics, Huntsville, AL), were used for LOH analysis. The locations of these markers and the *Smad2* and *Smad4* genes are shown in Fig. 1. PCR and electrophoresis conditions have been described elsewhere.[15]

PCR products eluted from the shifted bands detected by SSCP analysis were sequenced directly using a terminator cycle sequencing kit (Taq DyeDeoxy, Applied Biosystems, Foster City, CA) and an automated DNA sequencer (Model 373A, Applied Biosystems). In cases exhibiting LOH at 18q21.1, exon 11 of the *Smad4* gene was amplified and sequenced directly.

LOH at 18q21.1 was detected in 5 of 14 (35%) informative ESCC cases [*D18S46*, 2/12 (17%): *D18S467*, 4/12 (33%): *D18S460*, 4/10 (40%)] (Fig. 1). A deletion map for these cases is shown in Fig. 1. We previously demonstrated that LOH at the *DCC* locus occurs in 20% of ESCC using VNTR or RFLP markers.[16] The incidence of allelic deletion in the present study was higher at the centromeric position near the *Smad2* and *Smad4* genes than at the *DCC* gene locus. These results suggested that the *Smad2* and *Smad4* genes may be candidate tumor suppressor genes in ESCC. However, no mutation in the coding region of either gene was detected in 30 primary carcinomas and 7 cell lines. Only a base transition (G to A, nucleotide number[7] 1799) within the 3′-untranslated region of the *Smad4* gene was detected in a carcinoma (Fig. 2).

We examined various electrophoretic conditions, including concentration (0%–10%) of glycerol and temperature of SSCP (4°C or room temperature), in order to improve the sensitivity of the SSCP analysis.[18] In addition, exon 11 of the *Smad4* gene was directly sequenced in five cases which exhibited LOH at 18q21.1, because

the PCR products were longer than 400 bp. The lengths of the PCR products in the case of *Smad2* ranged from 236 to 301 bp, a size in which a pair base substitution can be detected with 90% reliability.[18] However, no intragenic mutations were detected.

Homozygous deletion is a more prevalent aberration of the *Smad4* gene than intragenic mutation,[7,13] as has also been reported for the *p16* gene.[17] Homozygous deletions of the *Smad4* gene have been detected in 25 (30%) of 84 cell lines and xenografts of pancreatic carcinomas,[7] 1 (50%) of 2 bladder[7] and 1 (13%) of 8 breast[13] carcinoma cell lines. However, there were no homozygous deletions in any of the examined cell lines derived from ESCC. Similarly, Kim *et al.*[14] failed to detect homozygous deletions in 20 primary head and neck squamous cell carcinomas (HNSCC) and 16 HNSCC cell lines, although they did find LOH in 7 of 15 (47%) at the *Smad4* locus. Moreover, Barrett *et al.*[19] noted frequent LOH without intragenic *Smad4* mutations in esophageal adenocarcinoma. There is obvious tumor specificity, since Schutte *et al.*[13] observed prevalent *Smad4* inactivation (48%) in pancreatic carcinomas, whereas it was uncommon ($<10\%$) in other tumor types. These results are concordant with the present study.

In view of the LOH results at 18q21.1, we anticipated that the *Smad2* gene rather than the *Smad4* gene might be a candidate tumor suppressor gene in ESCC. However, no intragenic mutation or homozygous deletion was detected in this gene. Mutations of the *Smad2* gene have been detected in colorectal carcinoma (11%, 2/18[10]; 6%, 4/66[11]), but Eppert *et al.*[11] screened for *Smad2* gene mutations in 101 breast carcinomas and 76 sarcomas, and did not detect any mutations. Uchida *et al.*[20] also reported a low incidence (2/57, 3%) of *Smad2* mutations in lung cancer. It remains possible that another target gene resides on 18q21.1.

The TGF$\beta$ family of proteins can cause cell cycle arrest, terminal differentiation, and apoptosis.[1,2] Hypermethylation and frame-shift mutations of the TGF$\beta$ type II receptor gene contribute in part to resistance against TGF$\beta$.[21,22] It is still unclear how the MAD-related proteins contribute to the disregulation of TGF$\beta$ pathways. Our results suggest that TGF$\beta$ resistance in ESCC is not related to disruption of *MAD*-related genes on 18q21.1. Recently, it has been shown that different TGF$\beta$ family members signal through distinct MAD isoforms.[6,8] Phosphorylation of *Smad1* (former name *MADR1*) is tightly regulated and rapidly induced by BMP2, but not by TGF$\beta$ or activin.[8] Thus, there are several candidate *MAD*-related genes which may be involved in this pathway. One such candidate is the homologous gene *JV5-1* on 5q31,[10] a region in which we have previously demonstrated frequent LOH in ESCC and gastric carcinomas.[15,23] Thus, further studies analyzing *JV5-1* mutations in ESCC are required.

This work was supported by Grants-in-Aid for Cancer Research (S8-1 and S8-13) from the Ministry of Health and Welfare of Japan, and a Grant-in-Aid (No. 08671479) from the Ministry of Education, Science, Sports and Culture of Japan.

*(Received January 17, 1997/Accepted February 21, 1997)*

REFERENCES

1) Filmus, J. and Kerbel, R. S. Development of resistance mechanisms to the growth-inhibitory effects of transforming growth factor-$\beta$ during tumor progression. *Curr. Opin. Oncol.*, **5**, 123–129 (1993).
2) 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).
3) Raftery, L. A., Twombly, V., Wharton, K. and Gelbart, W. M. Genetic screens to identify elements of the decapentaplegic signaling pathway in *Drosophila*. *Genetics*, **139**, 241–245 (1995).
4) Sekelsky, J. J., Newfeld, S. J., Raftery, L. A., Chartoff, E. H. and Gelbart, W. M. Genetic characterization and cloning of Mothers against dpp, a gene required for decapentaplegic function in *Drosophila melanogaster*. *Genetics*, **139**, 1347–1358 (1995).
5) Savage, C., Das, P., Finelli, A., Townsend, S., Sun, C., Baird, S. and Padgett, R. The *C. elegans* sam-2, sam-3 and sam-4 genes define a novel conserved family of TGF$\beta$ pathway components. *Proc. Natl. Acad. Sci. USA*, **93**, 790–794 (1995).
6) Graff, J. M., Bansal, A. and Melton, D. A. *Xenopus* Mad proteins transduce distinct subsets of signaling for the TGF$\beta$ superfamily. *Cell*, **85**, 479–487 (1996).
7) Hahn, S. A., Schutte, M., Hoque, A. T. M. S., Moskaluk, C. A., da Costa, L. T., Rozenblum, E., Weinstein, C. L., Fisher, A., Yeo, C. J., Hruban, R. H. and Kern, S. E. *DPC4*, a candidate tumor suppressor gene at human chromosome 18q21.1. *Science*, **271**, 350–353 (1996).
8) Hoodless, P. A., Haerry, T., Abdollah, S., Stapleton, M., O'Connor, M. B., Attisano, L. and Wrana, J. L. MADR1, a MAD-related protein that functions in BMP signaling pathways. *Cell*, **85**, 489–500 (1996).
9) Liu, F., Hata, A., Baker, J. C., Doody, J., Carcamo, J., Harland, R. M. and Massague, J. A human Mad protein acting as a BMP-regulated transcriptional activator. *Nature*, **381**, 620–623 (1996).
10) Riggins, G. J., Thiagalingam, S., Rozenblum, E., Weinstein, C. L., Kern, S. E., Hamilton, S. R., Willson, J. K. V., Markowitz, S. D., Kinzler, K. W. and Vogelstein, B.

MAD-related genes in the human. *Nat. Genet.*, **13**, 347–349 (1996).
11) Eppert, K., Schrer, S.W., Ozcelik, H., Pirone, R., Hoodles, P., Kim, H., Tsui, L.-C., Bapat, B., Gallinger, S., Andrulis, I. L., Thosen, G. H., Warana, J. L. and Attisano, L. MADR2 maps to 18q21 and encodes a TGFβ-regulated MAD related protein that is functionally mutated in colorectal carcinoma. *Cell*, **86**, 543–552 (1996).
12) Derynck, R., Gelbart, W. M., Harland, R. M., Heldin, C.-H., Kern, S. E., Massague, J., Melton, D. A., Mlodzik, M., Padgett, R. W., Roberts, A. B., Smith, J., Thomsen, G. H., Vogelstein, B. and Wang, X.-F. Nomenclature: vertebrate mediators of TGFβ family signals. *Cell*, **87**, 173 (1996).
13) Schutte, M., Hruban, R. H., Hedrick, L., Cho, K. R., Nadasdy, G. M., Weinstein, C. L., Bova, G. S., Isaacs, W. B., Cairns, P., Nawroz, H., Sidransky, D., Casero, Jr. R. A., Meltzer, P. S., Hahn, S. A. and Kern, S. E. *DPC4* gene in various tumor types. *Cancer Res.*, **56**, 2527–2530 (1996).
14) Kim, S. K., Fan, Y., Papadimitrakopoulou, V., Clayman, G., Hittelman, W. N., Hong, W. K., Lotan, R. and Mao, L. *DPC4*, a candidate tumor suppressor gene, is altered infrequently in head and neck squamous cell carcinoma. *Cancer Res.*, **56**, 2519–2521 (1996).
15) Ogasawara, S., Tamura, G., Maesawa, C., Suzuki, Y., Ishida, K., Sato, N., Uesugi, N., Saito, K. and Satodate, R. Common deleted region on the long arm of chromosome 5 in esophageal carcinoma. *Gastroenterology*, **110**, 52–57 (1996).
16) Maesawa, C., Tamura, G., Ogasawara, S., Suzuki, Y., Sakata, K., Sugimura, J., Nishizuka, S., Sato, N., Ishida, K., Saito, K. and Satodate, R. Loss of heterozygosity at the *DCC* gene locus is not crucial for the acquisition of metastatic potential in oesophageal squamous cell carcinoma. *Eur. J. Cancer*, **32A**, 896–898 (1996).
17) Maesawa, C., Tamura, G., Nishizuka, S., Ogasawara, S., Ishida, K., Terashima, M., Sakata, K., Sato, N., Saito, K. and Satodate, R. Inactivation of the CDKN2 gene by homozygous deletion and *de novo* methylation is associated with advanced stage esophageal squamous cell carcinoma. *Cancer Res.*, **56**, 3875–3878 (1996).
18) Hayashi, K. PCR-SSCP: a simple and sensitive method for detection of mutations. *Gene PCR Methods Appl.*, **1**, 34–38 (1991).
19) Barrett, M. T., Schutte, M., Kern, S. E. and Reid, B. J. Allelic loss and mutational analysis of *DPC4* gene in esophageal adenocarcinoma. *Cancer Res.*, **56**, 4351–4353 (1996).
20) Uchida, K., Nagatake, M., Osada, H., Yatabe, Y., Kondo, M., Mitsudomi, T., Masuda, A., Takahashi, To. and Takahashi, Ta. Somatic *in vivo* alterations of the *JV18-1* gene at 18q21 in human lung cancers. *Cancer Res.*, **56**, 5583–5585 (1996).
21) 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. W. Inactivation of the type II TGFβ receptor in colon cancer cells with microsatellite instability. *Science*, **268**, 1336–1338 (1995).
22) Garrigue-Antar, L. G., Souza, R. F., Vellucci, V. F., Meltzer, S. J. and Reiss, M. Loss of transforming growth factor-β type II receptor gene expression in primary human esophageal cancer. *Lab. Invest.*, **75**, 263–272 (1996).
23) Tamura, G., Ogasawara, S., Nishizuka, S., Sakata, K., Maesawa, C., Suzuki, Y., Terashima, M., Saito, K. and Satodate, R. Two distinct regions of deletion on the long arm of chromosome 5 in differentiated adenocarcinomas of the stomach. *Cancer Res.*, **56**, 612–615 (1996).