p53 Gene Mutations in Esophageal Cancer Detected by Polymerase Chain Reaction Single-strand Conformation Polymorphism Analysis

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Mutations of the p53 gene play an important role in the development of common human malignancies. We investigated mutations of this gene in 26 surgical specimens of esophageal cancer using the polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) analysis. The results were correlated with histological findings, DNA ploidy and the short-term relapse of the disease. PCR-SSCP analysis detected mutations of the p53 gene in 10 tumors (38%), eight in exons 5-6 and two in exons 7-8. A higher incidence of lymph node metastasis, poorly differentiated tumor, DNA aneuploidy and short-term relapse of the disease was observed in cases with p53 gene mutations, although the findings were not statistically significant.

Key words: Esophageal cancer — p53 gene — Polymerase chain reaction single-strand conformation polymorphism analysis

Recent progress in molecular genetics has revealed a consistent set of genetic alterations which may be related to multistep carcinogenesis. Although world-wide it is the sixth most common malignancy among males, there are few reports of genetic alterations in esophageal cancer; however, coamplification of *hst-1* and *int-2*^{1,2)} and amplification of the *c-myc* gene³⁾ have been found. It has recently been shown that the p53 gene is a tumor suppressor⁴⁾ and that its mutations play an important role in the development of many common human malignancies.⁵⁾ Frequent p53 gene mutations have been reported in diverse human tumors⁶⁻¹⁰⁾ including those of the alimentary tract; colorectal area,⁸⁾ stomach⁹⁾ and esophagus.¹⁰⁾ However, few reports have addressed the clinicopathological significance of mutations of this gene.

We applied the polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) method of analysis,^{11,12)} which is a rapid and highly sensitive method for detecting genetic aberrations, to the detection of p53 gene mutations in 26 surgical specimens of esophageal cancer. The presence of p53 gene mutations was correlated with the histological findings, DNA ploidy and short-term prognosis.

Tumor specimens were obtained from 26 patients with esophageal cancer who had undergone curative surgery at Iwate Medical University Hospital (Morioka) between 1990 and 1991. The specimens were immediately frozen after the surgery and stored at -80°C until analysis. Follow-up of these patients ranged from 4 to 22 months,

with a median of 11 months. The disease relapsed in 8 patients between 3 and 13 months after surgery (median of 9 months); 4 died. Three patients with a follow-up of less than three months, and one who died of causes other than esophageal cancer were excluded from prognostic evaluation. Thus, follow-up data were evaluated on 22 patients.

The histological findings of the 26 tumors were classified according to the Guide Lines for the Clinical and Pathologic Studies on Carcinoma of the Esophagus adopted by the Japanese Society for Esophageal Diseases (Table I).

DNA was isolated by proteinase K digestion and phenol/chloroform extraction. Oligonucleotide primers were synthesized by the phosphoramidite method with a DNA synthesizer (MilliGen/Bioscience, Division of Millipore, MA) and purified on Oligo-pak columns (MilliGen/Bioscience). The sequences of the primers used for PCR were as follows: 5'-TTCCTCTTCCTGCAGTACTCC-3' and 5'-AGTTGCAAACAGACC-TCA-3' for exons 5-6, 408 bp, and 5'-TCCTAGGTTG-GCTCTGAC-3' and 5'-CCTGCTTGCTTACCTC-GCT-3' for exons 7-8, 645 bp. A total of 100 ng of genomic DNA was amplified in 20 μ l of a buffer which was recommended by Perkin-Elmer Cetus, and which contained 1 mM MgCl₂ and 1 μ l of [α -³²P]dCTP (3000 Ci/mmol, 10 Ci/ml). Thirty-five cycles, each consisting of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min, were performed with a thermal programmer (Kurabo, Osaka). A volume of 2 μ l of the PCR product was diluted 100-fold with

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Table I. Histological Characteristics^{a)} of 26 Esophageal Cancers

Case	Age	Sex	Lymph node	Depth of invasion	Stage	Histological type
1	78	M	n2	a1	III	Well
2	68	M	n0	a3	IV	Mod
3	60	M	n3	a3	IV	Well
4	72	F	n3	a3	IV	Poor
5	66	M	n2	mp	III	Mod
6	59	M	n2	mp	III	Well
7	76	M	n3	a1	III	Well
8	61	M	n2	a2	III	Mod
9	74	M	n0	a0	II	Well
10	70	F	n3	a3	IV	Mod
11	58	M	n2	mp	III	Mod
12	53	M	n0	sm	I	Mod
13	79	F	n2	a2	III	Well
14	66	F	n2	a2	III	Well
15	65	M	n3	a1	IV	Well
16	75	M	n0	a3	IV	Well
17	57	M	n0	sm	0	Well
18	61	M	n2	a3	IV	Poor
19	47	M	n2	a3	IV	Poor
20	56	M	n4	mp	IV	Well
21	63	M	n4	a0	IV	Well
22	58	M	n4	a3	IV	Well
23	77	M	n3	mp	IV	Well
24	55	M	n0	mp	II	Mod
25	74	M	n2	mp	III	Mod
26	52	F	n0	mp	II	Well

a) Histological characteristics were classified based on the Guide Lines for the Clinical and Pathologic Studies on Carcinoma of the Esophagus adopted by the Japanese Society for Esophageal Diseases (1989).

a sequencing gel-loading buffer (98% deionized formamide, 10 mM EDTA pH 8.0, 0.025% xylene cyanol, 0.025% bromophenol blue), denatured at 80°C, and then applied (1 µl/lane) to a 6% neutral polyacrylamide gel. Electrophoresis was performed at 40 W for 3.5–5 h at 4°C. The gel was dried and exposed to Kodak XAR film at –80°C for 6–12 h. For direct DNA sequencing, the shifted band in exons 5–6 of a tumor (Case 6) detected by PCR-SSCP analysis was separated from the other PCR products, eluted from the polyacrylamide gel, and amplified by PCR using the same primers as used in prior PCR-SSCP analysis.¹³⁾ A double-stranded PCR product was purified with SUPREC™-02 (Takara Shuzo, Kyoto), and sequenced by the dideoxy chain termination method¹⁴⁾ with a 7-DEAZA Sequencing Kit, Version 2.0 (Takara Shuzo) as previously described.¹⁵⁾ The primers used for sequencing were 5'-TCTTCCTGCAGTACT-CCCT-3' and 5'-TGATTGCTCTTAGGTCTG-3'.

Fig. 1. PCR-SSCP analysis of exons 5–6 (A) and 7–8 (B) of the p53 gene. Lanes 1–26, esophageal cancers; lane 27, normal liver. Mobility shift is observed in lanes 4, 5, 6, 8, 13, 18, 20 and 25 of exons 5–6 (A), and in lane 16 and 26 of exons 7–8 (B). A band corresponding to a normal allele is not seen in lane 4 of exons 5–6 (A). Polymorphic variations of exons 7–8 (B) result from polymorphic base substitutions in intron 7 (see Ref. 16).

Eighteen of the 26 tumors were analyzed by DNA flow cytometry. Nuclei were isolated from frozen specimens with 0.1% Triton X-100 (Sigma Chemical, St. Louis, MO). The suspensions were treated with 0.1% RNase (Sigma), stained with 50 µg/ml propidium iodide (Sigma), and filtered through a 40 µm nylon mesh. DNA flow cytometry was performed using a FACScan (Becton Dickinson, Mountain View, CA).

Statistical significance of differences was evaluated by using the chi-square test with the criterion of $P < 0.05$.

Table II. Correlation of p53 Gene Mutation with Histological Findings, DNA Ploidy, and Short-term Relapse of the Disease

Parameters	p53 gene mutation		
	Positive	Negative	Total
Lymph node metastasis			
Positive	8 (80%)	11 (69%)	19 (73%)
Negative	2 (20%)	5 (31%)	7 (27%)
Depth of invasion			
sm, mp, a0	5 (50%)	7 (44%)	12 (46%)
a1	0 (0%)	3 (19%)	3 (12%)
a2	2 (20%)	1 (6%)	3 (12%)
a3	3 (30%)	5 (31%)	8 (30%)
Stage			
0, I, II	1 (10%)	4 (25%)	5 (19%)
III	5 (50%)	4 (25%)	9 (35%)
IV	4 (40%)	8 (50%)	12 (46%)
Histological type			
Well	5 (50%)	10 (63%)	15 (58%)
Mod	3 (30%)	5 (31%)	8 (31%)
Poor	2 (20%)	1 (6%)	3 (11%)
DNA ploidy			
Diploid	1 (11%)	4 (44%)	5 (28%)
Aneuploid	8 (89%)	5 (56%)	13 (72%)
Short-term relapse ^{a)}			
Relapsed	5 (63%)	3 (21%)	8 (36%)
Not relapsed	3 (37%)	11 (79%)	14 (64%)

a) Relapse in the period from 4 to 22 months.

A mobility shift was detected in 8 tumors in exons 5–6 by PCR-SSCP analysis (Fig. 1A). In one, a band corresponding to a normal allele was not seen (Fig. 1A), suggesting the deletion of this wild-type allele. Besides the polymorphic variations due to polymorphic base substitutions in intron 7,¹⁶⁾ a mobility shift was detected in 2 tumors in exons 7–8 (Fig. 1B). In one tumor, a direct DNA sequencing autoradiogram of exon 6 of the p53 gene revealed a single base deletion at the third position of codon 201, i.e., G deletion, resulting in a frame shift.

The correlation of the presence of p53 gene mutations with the histological findings is shown in Table II. Although there was higher incidence of lymph node metastasis (80%) and poorly differentiated tumor (20%), these findings did not achieve statistical significance ($P > 0.05$, chi-square test). DNA flow cytometry of 18 tumors demonstrated that 13 (72%) tumors were aneuploid and 5 (28%) were diploid. Eight of 9 tumors with p53 gene mutations were aneuploid (Table II). A short-term relapse of the disease occurred more frequently in the cases

with a mutated p53 gene (Table II), although without statistical significance ($P = 0.07$, chi-square test).

PCR-SSCP analysis is a highly sensitive method for detecting genetic aberrations; all the different point mutations of the *ras* gene in human tumor cell lines can be identified.¹⁷⁾ In the present analysis, p53 gene mutations were detected in 10 of the 26 (38%) esophageal cancers in exons 5–8, the most highly conserved regions, in agreement with a recent report on esophageal cancer in two high-incidence geographic areas.¹⁸⁾ Restriction fragment length polymorphism (RFLP) analysis of esophageal cancer¹⁹⁾ has revealed a chromosomal loss of heterozygosity of 17p, where the p53 gene has been mapped,²⁰⁾ at an incidence of 45%, which is similar to that found in our present analysis. The majority of the tumors with p53 mutations showed a loss of the remaining wild-type allele in colorectal tumor⁸⁾ and in gastric cancer.⁹⁾ These findings suggest that in cases with p53 gene mutations, the remaining wild-type allele may be deleted, and that the normal p53 gene function is also frequently inactivated in esophageal cancer.

Few reports have considered the clinicopathological significance of the p53 gene mutations. We noted a higher incidence of lymph node metastasis, poorly differentiated tumor and DNA aneuploidy in cases with a mutated p53 gene in this study, although this was not statistically significant. In gastric cancer, p53 gene mutations were detected only in aneuploid tumors,⁹⁾ and there was a significant correlation between the presence of these mutations and lymph node metastasis (unpublished data). A significant correlation between p53 overexpression and DNA aneuploidy has been reported in colorectal²¹⁾ and ovarian cancers.²²⁾ It has also been reported that esophageal cancers with a loss of 17p were often poorly differentiated histologically.¹⁹⁾ These findings are consistent with our present observations in esophageal cancer, and suggest that p53 gene mutations occur more frequently in aggressive cancers. In addition, a short-term relapse of the esophageal cancer occurred more frequently in the cases which carried a mutated p53 gene. In a recent report of p53 immunohistochemistry, a significant correlation between the overexpression of p53 and prognosis was identified in breast cancer.²³⁾

In conclusion, p53 gene mutations were frequently detected by PCR-SSCP analysis in esophageal cancer, and may be a prognostic factor in this disease. Further studies of a larger number of tumors are needed.

This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan, and by a Grant (No. 43) from the Keiryokai Research Foundation.

(Received February 21, 1992/Accepted April 4, 1992)

REFERENCES

- 1) Tsuda, T., Tahara, E., Kajiyama, G., Sakamoto, H., Terada, M. and Sugimura, T. High incidence of co-amplification of *hst-1* and *int-2* genes in human esophageal carcinomas. *Cancer Res.*, **49**, 5505-5508 (1989).
- 2) Kitagawa, Y., Ueda, M., Ando, N., Shinozawa, Y., Shimizu, N. and Abe, O. Significance of *int-2/hst-1* coamplification as a prognostic factor in patients with esophageal squamous carcinoma. *Cancer Res.*, **51**, 1504-1508 (1991).
- 3) Lu, S. H., Hsieh, L. L., Luo, F. C. and Weinstein, I. B. Amplification of the EGF receptor and *c-myc* genes in human esophageal cancers. *Int. J. Cancer*, **42**, 502-505 (1988).
- 4) Finlay, C. A., Hinds, P. W. and Levine, A. J. The p53 protooncogene can act as a suppressor of transformation. *Cell*, **57**, 1083-1093 (1990).
- 5) Nigro, J. M., Baker, S. J., Preisinger, A. C., Jessup, J. M., Hostetter, R., Cleary, K., Binger, S. H., Davidson, N., Baylin, S., Devilee, P., Glover, T., Collins, F. S., Weston, A., Modali, R., Harris, C. C. and Vogelstein, B. Mutations in the p53 gene occur in diverse human tumor types. *Nature*, **342**, 705-708 (1989).
- 6) Iggo, R., Gatter, K., Bartek, J., Lane, D. and Harris, A. L. Increased expression of p53 oncogene in primary lung cancer. *Lancet*, **335**, 675-679 (1990).
- 7) Bartek, J., Iggo, R., Gannon, J. and Lane, D. P. Genetic and immunohistochemical analysis of mutant p53 in human breast cancer cell lines. *Oncogene*, **5**, 893-899 (1990).
- 8) Baker, S. J., Preisinger, A. C., Jessup, J. M., Paraskeva, C., Markowitz, S., Willson, J. K. V., Hamilton, S. and Vogelstein, B. p53 gene mutations occur in combination with 17p allelic deletions as late events in colorectal tumorigenesis. *Cancer Res.*, **50**, 7717-7722 (1990).
- 9) Tamura, G., Kihana, T., Nomura, K., Terada, M., Sugimura, T. and Hirohashi, S. Detection of frequent p53 gene mutations in primary gastric cancer by cell sorting and polymerase chain reaction single-strand conformation polymorphism analysis. *Cancer Res.*, **51**, 3056-3058 (1991).
- 10) Hollstein, M. C., Metcalf, R. A., Welsh, J. A., Montesano, R. and Harris, C. C. Frequent mutation of the p53 gene in human esophageal cancer. *Proc. Natl. Acad. Sci. USA*, **87**, 9958-9961 (1990).
- 11) Orita, M., Suzuki, Y., Sekiya, T. and Hayashi, K. Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. *Genomics*, **5**, 874-879 (1989).
- 12) Orita, M., Iwahara, H., Kanazawa, H., Hayashi, K. and Sekiya, T. Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. *Proc. Natl. Acad. Sci. USA*, **86**, 2766-2770 (1989).
- 13) Suzuki, Y., Sekiya, T. and Hayashi, K. Allele-specific polymerase chain reaction: a method for amplification and sequence determination of a single component among a mixture of sequence variants. *Anal. Biochem.*, **192**, 82-84 (1991).
- 14) Sanger, F., Nicklen, S. and Coulson, A. R. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA*, **74**, 5463-5467 (1977).
- 15) Kuskawa, N., Uemori, T., Asada, K. and Kato, I. Rapid and reliable protocol for direct sequencing of material amplified by the polymerase chain reaction. *BioTechniques*, **9**, 66-72 (1990).
- 16) Okamoto, A., Sameshima, Y., Yokoyama, S., Terashima, Y., Sugimura, T., Terada, M. and Yokota, J. Frequent allelic losses and mutations of the p53 gene in human ovarian cancer. *Cancer Res.*, **51**, 5171-5176 (1991).
- 17) Suzuki, Y., Orita, M., Shiraishi, M., Hayashi, K. and Sekiya, T. Detection of *ras* gene mutations in human lung cancers by single-strand conformation polymorphism analysis of polymerase chain reaction products. *Oncogene*, **5**, 1037-1043 (1990).
- 18) Hollstein, M. C., Peri, L., Mandard, A. M., Welsh, J. A., Montesano, R., Metcalf, R. A., Bak, M. and Harris, C. C. Genetic analysis of human esophageal tumors from two high incidence geographic areas: frequent p53 base substitutions and absence of *ras* mutations. *Cancer Res.*, **51**, 4102-4106 (1991).
- 19) Wagata, T., Ishizaki, K., Imamura, M., Shimada, Y., Ikenaga, M. and Tobe, T. Deletion of 17p and amplification of the *int-2* gene in esophageal carcinomas. *Cancer Res.*, **51**, 2113-2117 (1991).
- 20) McBride, O. W., Merry, D. and Glivol, D. The gene for human p53 cellular tumor antigen is located on chromosomes 17 short arm (17p13). *Proc. Natl. Acad. Sci. USA*, **83**, 130-134 (1986).
- 21) Remvikos, Y., Laurent-Puig, P., Salmon, R. J., Frelat, G., Dutrillaux, B. and Thomas, G. Simultaneous monitoring of p53 protein and DNA content of colorectal adenocarcinomas by flow cytometry. *Int. J. Cancer*, **45**, 450-456 (1990).
- 22) Marks, J. R., Davidoff, A. M., Kerns, B. J., Humphrey, P. A., Pence, J. C., Dodge, R. K., Clarke-Pearson, D. L., Iglehart, J. D., Bast, R. C., Jr. and Berchuck, A. Overexpression and mutation of p53 in epithelial ovarian cancer. *Cancer Res.*, **51**, 2979-2984 (1991).
- 23) Iwaya, K., Tsuda, H., Hiraide, H., Tamaki, K., Tamakuma, S., Fukutomi, T., Mukai, K. and Hirohashi, S. Nuclear p53 immunoreaction associated with poor prognosis of breast cancer. *Jpn. J. Cancer Res.*, **82**, 835-840 (1991).