Non-radioactive Screening of p53 Mutations in Human Oral Cancers Detected by Single Strand Conformation Polymorphism Analysis: Comparison with the Protein Accumulation

Tetsuyo ODAJIMA[1)3)*], Akira YAMAGUCHI[1)], Itaru NAGAI[1)], Gen-iku KOHAMA[1)]
Masa-aki SATOH[2)], Toshiharu YAMASHITA[3)] and Kei FUJINAGA[3)]

1) *Department of Oral Surgery, Sapporo Medical University School of Medicine, South 1, West 16, Chu-oku, Sapporo 060, JAPAN*
2) *Division of Clinical Pathology, Sapporo Medical University Hospital, Sapporo Medical University School of Medicine, South 1, West 16, Chu-oku, Sapporo 060, JAPAN*
3) *Department of Molecular Biology, Cancer Institute, Sapporo Medical University South 1, West 16, Chu-oku, Sapporo 060, JAPAN*

Key words: p53 gene mutation, Oral cancer, Clinicopathological variable

ABSTRACT

p53 gene mutation and its protein accumulation are widely detected in human cancers and have prognostic significance. To examine the relationship between p53 mutation and its protein accumulation, tumor samples from 65 oral cancer patients were analyzed using both single strand conformational polymorphism (SSCP) technique to screen the presence of p53 gene mutations and immunohistochemistry to detect the p53 protein accumulations. Results were simultaneously associated with clinicopathological variables of the cancers and prognoses of the patients. p53 gene mutations were found in 18 cancers (7.6%), whereas aberrant accumulations of p53 protein were present in 31 cancers (47.6%). All of the oral cancers with p53 mutation showed positive immunoreactivity, but in addition, p53 protein accumulations were still found in 13 oral cancers (20%) without detectable gene mutations. The concordance between the protein expression and the SSCP analysis was 80%. Such p53 abnormalities were significantly correlated with lymph node metastasis and clinical stage. Patients with the detectable p53 abnormalities survived for a significantly shorter period of time. p53 abnormalities are reliable as an indicator for evaluating malignant potential of oral cancers.

INTRODUCTION

Until now, lymph node metastasis has been the most generally accepted prog-

* To whom requests for reprints should be addressed

nostic factor used as an indicator for treatment decisions in human cancers. Other efforts have been undertaken to establish new prognostic factors or even to create reliable prognostic indices that could improve clinical estimates in individual patients. Tumor suppressor genes play important roles in the regulation of cell growth and differentiation and the altered tumor suppressor genes relate to the cancer development or progression (1). Mutation of p53 tumor suppressor gene and the accumulation of its aberrant protein product within the nucleus are the most common alterations related to the pathogenesis of various human cancers (2,3). Moreover, p53 mutations and/or its protein accumulations are correlated with cancer progression, suggesting its possibility as important prognostic indicators (4,5).

There are two methods for the detection of p53 gene mutation. SSCP method introduced by Orita et al. is a rapid and sensitive technique for detecting single-base mutations within a DNA sequence (6). Many investigations have been made to detect p53 gene mutation in a variety of human cancer using this method (7-11). On the other hand, it is well known that the wild-type p53 gene protein functions as a suppressor of tumor growth, whereas mutated p53 protein may inactivate the wild-type p53 function, resulting in cell transformation (12). Mutation at the p53 locus may lead to the synthesis of aberrant mutant p53 protein with a prolonged half-life and increased stability (13). This accumulated protein is the target of p53 gene mutation simply-detectable at the protein level. However, the result of p53 protein accumulation is not always in agreement with the p53 mutation at gene level (8-10). In the present study, we examined aberrant accumulation of p53 protein in oral cancers and SSCP analysis to see whether these two methods correlated with each other. Results obtained were simultaneously analyzed in association with clinicopathological variables of cancers examined and prognoses of the cases in order to evaluate the relevance of p53 gene abnormality as a prognostic indicator.

MATERIALS AND METHOD

Tissue Samples

To ascertain a possible relationship between p53 abnormality clinicopathological variables and patient prognosis, analyses were carried out for 65 patients who had undergone complete tumor resection and for whom follow-up data were available in this study. A portion of each biopsied cancer specimen from the patients with oral cancer was snap frozen in liquid nitogen and stored at $-80°C$ until PCR-SSCP analysis for p53 gene mutation. Another portion was fixed in buffered formalin, and embedded in paraffin for routined histological examination and immunohistochemistry. Five normal oral mucosal specimens were also

examined as control.

Immunohistochemistry for p53 protein accumulation

4 μm histological sections were cut from the formalin-fixed and paraffin-embedded tissue blocks for p53 protein immunohistochemistry. The deparaffinized sections were pretreated by autoclaving in citrate buffer (pH 6.0) at 120°C for 5 min. After standard inhibition of endogenous peroxidase activity, staining of p53 protein for the tissue sections was performed using Histofine ABC immunoperoxidase kit system(Nichirei, Japan) and DO7 p53 monoclonal antibody(Novocastra, Britain). Finally, the histological sections were counter-stained with hematoxilin. Results were evaluated by a semiquantitative method: p53 protein expressions with more than 30% staining tumor cells were evaluated as positive.

DNA Extraction

Samples from the frozen tissue specimens were homogenized in cold TNM extraction buffer containing 20 mM Tris-HCl (pH 7.5), 0.1 M NaCl and 1.5 mM $MgCl_2$. DNA extraction was performed using a Sepagene DNA extraction kit (Wako, Japan). The DNA collected by ethanol precipitation was air-dried, resuspended in TE buffer containing 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA, and finally the concentration was measured using a spectrophotometer. The extracted DNA was kept at 4°C until use for experiment.

PCR DNA amplification

The oligonucleotide primers used for p53 PCR amplification were designed on the basis of published sequences as shown in Table 1 (14). Each 0.5 μg of genomic DNA extracted from oral cancer tissue was added to a total volume of 50 μl of reaction mixture containing 10 mM Tris-Hcl (pH 8.3), 1.5 mM $MgCl_2$, 300 pM of 3′ and 5′ primers for each exon of the p53 gene, 200 μM dNTPs and 2.5 units of Taq DNA polymerase (Takara, Japan). PCR was carried out in a

Table 1 Oligonucleotide primer sequences

Exon 5(325 bp)	sense	5'-TTCCTCTTCCTGCAGTACTC-3′
	antisense	5'-CCCAGCTGCTCACCATCG-3′
Exon 6(236 bp)	sense	5'-CCTCACTGATTGCTCTTAGG-3′
	antisense	5'-AGTTGCAAACCAGACCTCAG-3′
Exon 7(139 bp)	sense	5'-GCGTGTTGCCTCCTAGGTTG-3′
	antisense	5'-CAAGTGGCTGCCTGACCTGGA-3'
Exon 8-9(330 bp)	sense	5'-CCTATCCTGAGTAGTGGTAA-3'
	antisense	5'-CCAAGACTTAGTACCTGAAG-3′

thermal cycler (Perkin-Elmer Cetus, USA) for 40 cycles at 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min. After the last cycle of amplification, the extension was continued for an additional 7 min at 72°C. After amplification, 10 μl of PCR product at each exon was electrophoresed on 3% agarose-gel for confirmation of amplification of p53 genomic DNA.

p53 SSCP Analysis

Each PCR product of p53 gene (2 μl) was added to 9 μl of loading buffer (95% formamide, 10 mM NaOH, 0.05% bromophenol blue, and 0.05% xylene cyanol), heated at 94°C for 2 min, and chilled on ice. A 5 μl aliquot of the sample mixture was electrophoresed through non-denaturing MDE gel (AT Biochem., USA), at 50 W for 3 to 5 hr under cooling. After electrophoresis, the gel was silver-stained using silver stain kit (Bio-Rad, USA) according to the manufacturer's instruction for the detection of a mobility shift band suggesting mutation of the p53 gene.

Statistical Analysis

Results obtained from these analyses were statistically analyzed with Chi-square tests in association with clinicopathological variables (tumor size, lymph node status, clinical disease stage, cancer differentiation, pathological mode of cancer invasion) and patient prognoses. P-values of less than 0.05 were considered statistically significant. Cumulative survival rate for clinical outcome were made on the basis of the Kaplan-Meier method (15). Statistical significance of the survival rate differences was tested by log-rank.

RESULTS

p53 protein accumulation

In this study, an antibody DO7 was used as a primary antibody against p53 protein for standard ABC immmunohistochemistry, because this antibody is the most suitable (16). Immunoreactive p53 protein was localizeed only in the nuclei of cancer cells, not in controlled normal oral squamous epithelia. The aberrant nuclear accumulations of p53 protein were found in 31 (47.7%) of the 65 oral cancers examined (Fig. 1).

p53 gene mutation and the correlation with aberrant accumulation of p53 protein

In the 65 oral cancers included in this study, exons 5 to 9 of the p53 gene were examined for mutations by the PCR-SSCP method, since these exons are the ones most frequently involved in mutational events of the p53 gene (7-11, 14, 17). In this study we made a non-radioactive analysis to detect p53 gene muta-

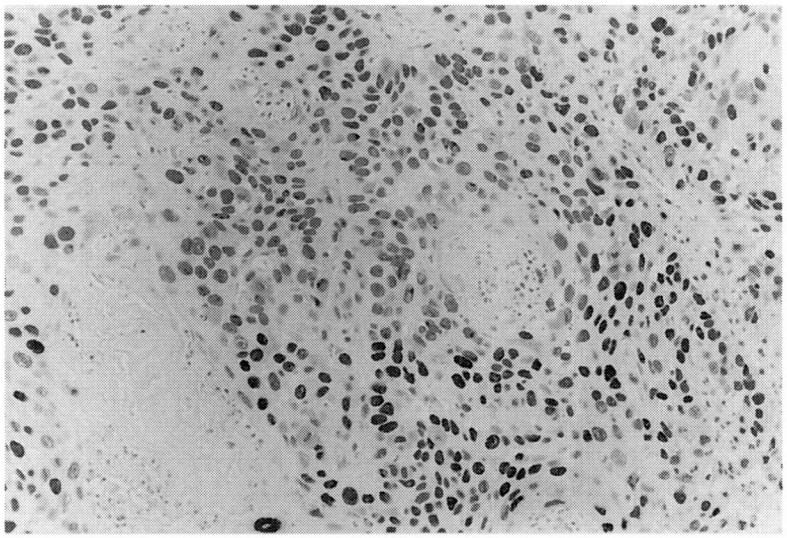

Fig. 1 Nuclear aberrant overexpressions of p53 protein in cancer cells from a well differentiated squamous cell carcinoma. x200. Approximately 67% of cancer cells showed positive immunoreactivity.

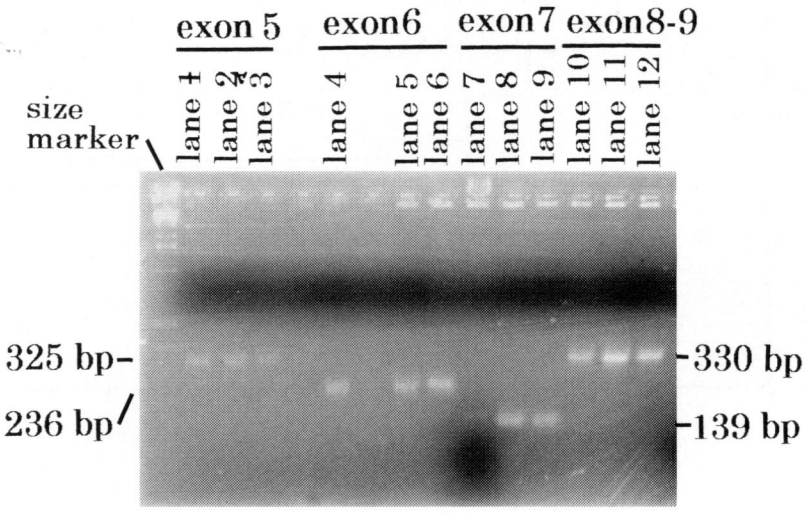

Fig. 2 PCR amplification of p53 gene in a tumor cell line and oral cancers.
Each amplified p53 gene is found as a single band at the corresponding level of size in exons 5, 6, 7 and 8-9 on 3% agarose gel. Lanes 1, 4, 7 and 10 are from HSC-3 tumor cell line, lanes 2, 5, 8 and 11 are from case 6, and lanes 3, 6, 9 and 12 are from case 7.

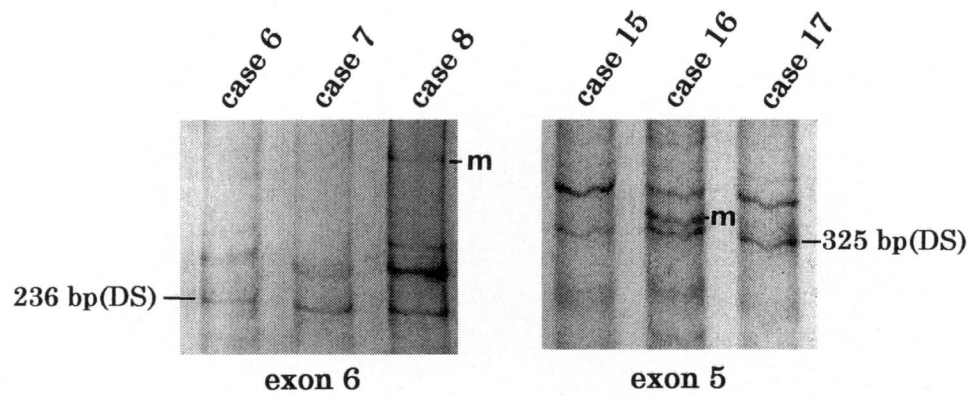

Fig. 3 SSCP analyses in exons 5 and 6 of the p53 genes extracted from cases 6, 7, 8 15, 16, 17. m : mutated mobility shift band, DS : double strand band.

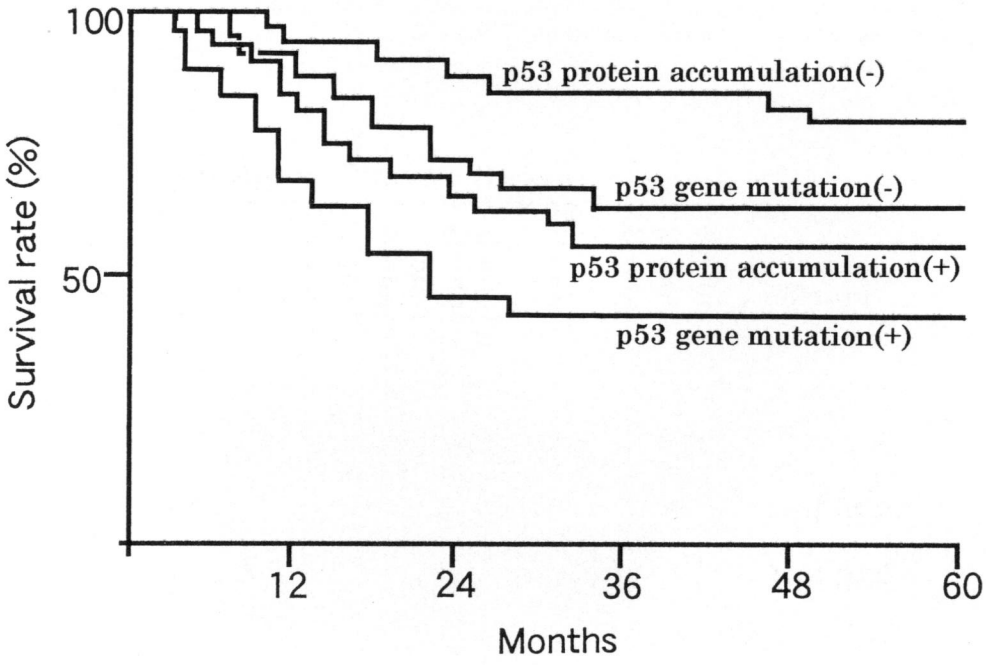

Fig. 4 Kaplan-Meier survival curves after curative resection of oral cancer based on the presence or absence of p53 abnormalities. p53 protein accumulation(−) vs p53 protein accumulation(+) : p=0.015, p53 gene mutation(−) vs p53 gene mutation(+) : p=0.0039.

tions, because non-radioactive silver-staining for the detection of p53 mutations is a sensitive, rapid, and simple technique that is comparable to the standard radioactive techniques (18). Fig. 1 shows PCR amplifications of p53 genes in human oral cancer specimens using exons 5 to 9 as primers. Genomic DNAs extracted from oral cancer specimens were well amplified by PCR and visualized as single band at each lane on a 3% agarose gel. In the SSCP analyses using non-denaturing MDE gel, bands with different mobility shift were found in 18 of the 65 oral cancers (27.7%) examined, which indicated the existence of DNA conformation abnormalities corresponding to p53 gene mutations. On the other hand, none of the 5 normal oral mucosal specimens examined showed any band with different mobility. Six of the 18 p53 gene mutations were detected in exon 5 (33.3%), nine in exon 6 (50%), three in exon 7 (16.7%), but none in exon 8-9.

Table 2 shows the correlation of p53 gene mutation with its protein accumulation. Although all of the 18 oral cancers with p53 mutation were also positive for its protein expression, 13 of 47 oral cancers with no p53 mutation were still positive for p53 protein expression. Thirty four of the 65 oral cancers showed no positive reaction in either analysis. Thus, there was a complete concordance between p53 gene abnormalities in 52 oral cancers that contained both positive and/or negative ones in both analyses.

Correlation of p53 abnormalities with clinicopathological variables in oral cancer and patient prognosis

The association of p53 gene abnormalities (aberrant protein accumulation and gene mutation) with the clinicopathological variables (tumor size, lymph node metastasis, clinical disease stage, differentiation and pathological mode of invasion) is detailed in Table 3. The p53 abnormalities were closely related to lymph node metastasis and clinical stage with a significantly-higher incidence of p53 abnormalities in cases with lymph node metastasis and advanced clinical stage ($p<0.05$), but p53 abnormalities did not relate significantly to tumor size, differentiation and pathological mode of invasion. As shown in Fig. 6, the Kaplan-Meier survival curve demonstrated that patients with p53 abnormalities sur-

Table 2 Relationship between p53 gene mutation and p53 protein accumulation in oral cancers

		p53 protein accumulation	
	No. of case	positive(%)	negative(%)
p53 gene mutation			
positive	18	18(27.6%)	0(%)
negative	47	13(20.0%)	34(52.3%)

Table 3 p53 protein expression, p53 gene mutation and clinicopathological variables in oral cancers ★$p<0.05$

Variable		No. of cases	p53 abnormality	
			p53 protein positive (%)	p53 mutation positive (%)
Tumor size				
	<3cm	36	12 (18.4%)	7 (10.7%)
	≧3cm	29	19 (29.2%)	11 (16.9%)
Lymph node metastasis				
	negative	37	10 (15.3%)	6 (9.2%)
	positive	28	21 (32.3%) ★	12 (18.4%) ★
Clinical stage				
	I + II	31	9 (9.3%)	5 (7.7%)
	III + IV	34	22 (33.8%) ★	13 (20.0%) ★
Differentiation				
	well	29	14 (21.6%)	6 (9.2%)
	moderate	25	16 (24.6%)	8 (12.3%)
	poor	11	8 (12.3%)	4 (6.1%)
Pathological mode of invasion				
	1 + 2	16	8 (12.3%)	4 (6.1%)
	3	31	14 (21.5%)	8 (12.3%)
	4	18	9 (13.8%)	7 (10.7%)

vived for a significantly shorter period of time, particularly showing the lowest survival rate in patients with p53 gene mutations ($p=0.0039$). Each survival rate after 5 years is 43.7% in patients with the p53 gene mutation, 56.2% with p53 protein accumulation, 63.5% without the p53 gene mutation and 78.9% without p53 protein accumulation.

DISCUSSION

The primary aim of this investigation is to determine whether the presence of p53 genetic alterations could serve to identify a group of patients who would experience a distinct outcome after complete resection of the cancers. To this aim, we examined whether immunohistochemical accumulation of p53 protein in oral cancers is reliable and sensitive as an indicator of p53 gene mutation and whether these abnormalities of p53 gene are useful as new prognostic indicators for an estimation of the biological behavior of cancer before treatment.

We first carried out immunohistochemical analyses of p53 protein. Gener-

ally, there are some problems in immunohistochemical analysis. One is the presence of false negative cases due to the masking of immunoreactivity in p53 protein. To remedy this problem, we coped with overheating histological sections before immunohistochemical procedure for antigen retrieval of p53 protein. Another problem is that positive cases included tissues that contained only solitary cell clusters with positive nuclear staining. It is conceivable that these heterogeneous staining patterns are paralleled by genetic heterogeneity in a minority of tumor cell (19). In fact, in many reports, expressions of p53 protein were assessed as positive cases when more than 10% staining tumor cells were observed (9, 16). In this study, only cases with more than 30% staining cancer cells were evaluated as positive to maintain a more strict estimation, and 31 (47.6%) of 65 oral cancers examined were positive for the aberrant accumulation expression of p53 protein. Antibody DO7 used in this study recognizes wild-type as well as mutant forms of p53 protein. As only mutant forms of p53 protein are detectable by immunohistochemistry, it is suggested that appreciable mutations of p53 are present in such 31 oral cancers with p53 protein accumulations.

In contrast to the nuclear accumulation of p53 protein, p53 gene mutations were found in 18 (27.6%) of the 65 oral cancers with PCR-SSCP analysis. The incidences of p53 abnormalities including p53 protein acumulation and its gene mutation agree roughly with those in many tumors including oral cancer, as determined either by direct characterization of the genetic alteration or indirectly by immunohistochemistry to p53 antibodies (7-11, 14, 17). All of the 18 oral cancers with p53 gene mutation were also immuno-positive for p53 protein, showing the usefulness of immunohistochemistry for the detection of p53 gene mutation in these oral cancers. However, we observed positive expressions of p53 protein in 13 of the 47 cancers without a detectable p53 gene mutation. The concordance of the results for immunohistochemistry and PCR-SSCP was 80% including both positive cases and negative cases, with 20% of cases discordant. The lowered incidence (27.6%) of p53 gene mutation compared with that (47.6%) of aberrant accumulation of p53 protein may result from false negative cases produced by SSCP analysis of the p53 gene, although it does have good specificity. The discrepancy may be due to low tumor cellularity and the heterogeneity of specimens examined for the analysis of PCR-SSCP, or to the differences in the sensitivities of PCR-SSCP and immunohistochemistry. Another possibility is that mutations might occur in exons other than those examined in this study. Recently, intriguing studies suggest that aberrant accumulations of p53 protein do not always indicate its gene mutation, and other mechanisms are related to the p53 protein accumulation (20). However, our findings do not deny the significance of p53 protein accumulation as an indicator of p53 gene mutation.

p53 abnormalities including nuclear protein accumulation and gene mutation were closely correlated with positive lymph node metastasis and advanced clinical stage. Lymph node metastasis is an important factor for the determination of clinical stage as well. These findings strongly suggest that p53 abnormalities might be related to lymph node metastasis. Furthermore, the Kaplan-Meier survival curve revealed that when all patients were analyzed, p53 abnormalities shortened significantly their survival period. An association of p53 abnormalities with a poor prognosis after surgical resection, similar to the results in human oral cancers examined in this study, has been also reported for gastric cancer (21), ovarian cancer (7) and colon cancer (11), although other types of human cancer were found to have no such association (9, 22, 23). Although p53 gene mutation is generally considered to be associated with carcinogenesis, our findings strongly suggests that it may also be a late event in the progression of oral cancers in association with advanced stage, lymph node metastasis and/or the behavior of cancer malignancy.

Although some problems remain to be determined in this study, p53 abnormalities are of clinical usefulness for evaluation of tumor progression and prognosis. Improvement of the sensitivity in combined assay with immunohistochemistry and SSCP methods may give us further useful information regarding treatment and management of cancer patients. Retrospective analysis is also possible using non-RI PCR-SSCP for genomic DNA extracted from formalin-fixed and paraffin-embedded specimens to examine a large number of cases. Qualitative analysis of p53 gene mutations is also needed as well as direct sequential study.

REFERENCE

1. BISHOP JM, Molecular themes in oncogenesis. Cell 1991, 64: 235-248.
2. VOGELESTEIN B. CANCER. A deadly inheritance. Nature 1990, 348: 681-682.
3. HOLLSTEIN M, SIDRANSKY D, VOGELSTEIN B, HARRIS CC. p53 mutations in human cancers. Science 1991, 253: 49-53.
4. PORTER PL, GOWN AM, KRAMP SG, COLTRERA MD. Widespread p53 overexpression in human malignant tumors. An immunohistochemical study using methacarn-fixed, embedded tissue. Am J Pathol 1992, 140: 145-153.
5. HALL PA, RAY A., LEMOINE NR, MIDGLEY CA, KRAUSZ T, LANE DP. p53 immunostaining as a marker of malignant disease in diagnostic cytopathology. Lancet 1991, 338: 513-518.
6. ORITA M, SUZUKI Y, SEKIYA T, HAYASHI K. Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. Genomics 1989, 5: 874-879.
7. Niwa K, ITOH M, MURASE T, MORISHITA S., ITOH N., MORI H, TAMAYA T. Alteration of p53 gene in ovarian carcinoma: clinicopathological correlation and prognostic

significance. Br J Cancer 1994, 70: 1191-1197.
8. NYLANDER K, NILSSON P, MEHLE C, ROOS G. p53 mutations, protein expression and cell proliferation in squamous cell carcinomas of the head and neck. Br J Cancer 1995, 71, 826-830.
9. BOSARI S, VIALE G., RONCALLI G, GRAZIANI D, BORSANI G., LEE, AKC , Coggi G. p53 gene mutations, p53 protein accumulation and compartmentalization in colorectal adenocarcinoma. Am J Pathol 1995, 147: 790-798.
10. ATULA S, KURVINEN R, GRENMAN R, SYRJANEN S. SSCP pattern indicative for p53 mutation is related to advanced stage and high-grade of tongue cancer. Oral Oncol, Eur J Cancer 1996, 4: 222-229.
11. SMITH DR, JI CY, GOH HS. Prognostic significance of p53 overexpression and mutation in colorectal adenocarcinomas. Br J Cancer 1996, 74: 216-223.
12. LEVINE AJ, MOMAND J, FINLAY CA. The p53 tumor suppressor gene. Nature 1991, 351: 453-456.
13. IGGO R, GATTER K, BARTEK J. Increased expression of mutant forms of p53 oncogene in primary lung cancer. Lancet 1990,335: 675-679.
14. MURAKAMI Y, HAYASHI K, HIROHASHI E, SEKIYA T. Aberrations of the tumor-suppressor p53 and retinoblastoma genes in human hepatocellualr carcinomas. Cancer Res 1991, 51: 5520-5525.
15. KAPLAN EL, MEIYER P. Nonparametric estimations for incomplete observations. J Am Stat Assoc 1956, 53: 457-481.
16. Righetti SC, TORRE GD, PILOTTI S, MENARD S, OTTONE F, COLNAGHI MI, PIEROTTI MA, LAVARINO C, CORNAROTTI M, ORIANA S, BOHM S, BRESCIANI GL, SPATTI G, ZUNINO F. A comparative study of p53 gene mutations, protein accumulation, and response to cisplatin-based chemotherapy in advanced ovarian carcinoma. Cancer Res 1996, 56: 689-693.
17. NEES M, HOMANN N, DISCHER H, ANDL T, ENDERS C, HEROLD-MENDE C, SCHUMANN A, BOSCH FX. Expression of mutated p53 occurs in tumor-distant epithelia of head and neck cancer patients: A possible molecular basis for the development of multiple tumors. Cancer Res 1993, 53: 4189-4196.
18. BOSARI S, MARCHETTI A, BUTTITTA F, GRAZIANI D, BORSANI G, LODA M, BEVILACQUA G, COGGI G. Detection of p53 mutations by single-strand conformation polymorphisms (SSCP) gel electrophoresis. A comparative study of radioactive and nonradioactive silver-stained SSCP analysis. Diag Mol Pathol 1995,4: 249-255.
19. CHEN LC, KURISU W, LIJUNG BM, GOLDMAN ES, MOORE D, SMITH HS. Heterogeneity for allele loss in human breast cancer. J Natl Cancer Inst 1992, 84: 506-510.
20. WYNFORD-THOMAS D. p53 in tumor pathology: can we trust immunocytochemistry? J Pathol 1992, 166: 329-330.
21. MARTIN HM, FILLIPE MI, MORRIS RW, LANE DP, SILVESTRE F. p53 expression and prognosis in gastric carcinoma. Int J Cancer 1992, 50: 859-862.
22. SCOTT N, SAGAR P, STEWART J, BLAIR GE, DIXON MF, QUIRKE P. p53 in colorectal cancer: clinicopathological correlation and prognostic significance. Br J Cancer 1991, 63: 317-319.

23. MCLAREN R, KUZU I, DUNNILL M, HARRIS A, LANE D, GATTER KC. The relationship of p53 immunostaining to survival in carcinoma of the lung. Br J Cancer 1992, 66: 735-738.