Accumulation of the p53 tumor-suppressor gene product in oral leukoplakia

MARK W. WOOD, MD, JESUS E. MEDINA, MD, GLENN C. THOMPSON, PhD, JOHN R. HOUCK, MD, and KYUNG-WHAN MIN, MD, Oklahoma City, Oklahoma

OBJECTIVES: (1) To determine whether the protein of the suppressor gene p53 accumulates in leukoplakia of the oral cavity in individuals who use snuff; and (2) to determine whether a correlation exists between the accumulation of p53 protein and the degree of epithelial dysplasia present in oral leukoplakia.

DESIGN: Retrospective analysis of archival tissue specimens.

SETTING: The University Hospital, a tertiary referral hospital affiliated with the Oklahoma University Medical Center, Oklahoma City, Oklahoma.

PATIENTS: In the first part of the study, biopsy specimens of leukoplakia from 12 persons who used snuff were compared with specimens from uninvolved oral mucosa of the same persons and with biopsy specimens from 12 nontobacco-using persons. In the second part of the study, accumulation of p53 protein was determined in 42 archival paraffin-embedded specimens from oral leukoplakia and correlated with the degree of epithelial dysplasia.

METHODS: Accumulation of p53 protein was assessed by immunoperoxidase staining with four different primary antibodies. Positive cells were counted in five consecutive high-power fields.

RESULTS: In part one, the average number of positive cells in the leukoplakia of snuff-users (21.89 ± 4.33; mean ± SE) was higher than that of normal-appearing mucosa (4.00 ± 1.0; $p < 0.05$) and that of nontobacco-using controls (7.00 ± 5.04). In part two, the average number of positive cells was higher in the moderately dysplastic (140.36 ± 30.03) and severely dysplastic lesions (232.86 ± 26.85) than in the mildly dysplastic lesions (14.53 ± 3.33; $p < 0.05$). The correlation between the degree of epithelial dysplasia and the number of cells positive is strong (Spearman's correlation coefficient = 0.853).

CONCLUSIONS: The accumulation of p53 protein in leukoplakia of snuff-users is higher than in normal-appearing oral mucosa from both snuff-users and nontobacco-using controls. A strong correlation exists between the degree of epithelial dysplasia present in oral leukoplakia and the number of cells staining positive for p53. The accumulation of p53 protein holds potential as an intermediate end point in studies of chemoprevention of oral cancer. (OTOLARYNGOL HEAD NECK SURG 1994;111:758-63.)

Leukoplakia of the buccal and gingival mucosa is found in as many as 50% to 60% of persons who use snuff.[1-3] Oral leukoplakia is considered to be a premalignant lesion by most clinicians[4-6] because squamous cell carcinoma eventually occurs in 3% to 18% of persons who have it.[7-9]

The development of carcinoma in oral leukoplakia usually occurs over a period of several years. Thus it is desirable to identify biomarkers that would be useful for monitoring both the risk of malignancy developing in oral leukoplakia and the efficacy of prevention interventions. These biomarkers are described as "intermediate" because it is hoped that they will allow detection of precancerous changes before carcinoma is present. Investigations of such biomarkers in oral leukoplakia have included proliferation markers (mitotic frequency), epithelial markers of differentiation (transglutaminase I and involucrin), and genomic markers (DNA ploidy, micronuclei, and chromosome specific probes).[10] Gilchrist et al.[11] demonstrated that epithelial markers of

From the Departments of Otorhinolaryngology (Drs. Wood, Medina, Thompson, and Houck) and Pathology (Dr. Min), University of Oklahoma.

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Reprint requests: Jesus E. Medina, MD, Chairman, Department of Otorhinolaryngology, University of Oklahoma, Rm. 3SP-226, P.O. Box 26901, Oklahoma City, OK 73190.

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differentiation would not be useful as intermediate biomarkers, and the usefulness of other differentiation and genetic markers has not been adequately evaluated.[12]

Accumulation of the tumor suppressor gene p53 protein holds potential as a biomarker in the study of leukoplakia. The p53 gene has been identified as a tumor-suppressor gene in its normal expression.[13] Mutation of the p53 gene is currently the most commonly identified gene mutation associated with human neoplasia.[14] Normally the protein product has a very short half-life and cannot be identified by standard immunohistochemical techniques. However, mutation of the gene frequently results in accumulation of a protein product that can be identified immunohistochemically.[13] Furthermore, accumulation of p53 protein has been found in premalignant lesions of the breast,[15] colon,[16] esophagus,[17] and larynx,[18] as well as in the dysplastic epithelium adjacent to carcinomas of the breast and esophagus.[15,19] To date, accumulation of p53 protein has been reported in only two cases of oral leukoplakia.[20] Thus the usefulness of p53 mutation as a predictor of malignant transformation has not been investigated thoroughly in regard to oral leukoplakia.

This study was designed with the following objectives in mind: first, to determine whether accumulation of tumor-suppressor gene p53 protein occurs in leukoplakia of the oral cavity in persons who use snuff; and second, to determine whether a correlation exists between the accumulation of p53 protein and degree of epithelial dysplasia present in oral leukoplakia.

METHODS AND MATERIAL

Collection of Patient Material

The first part of the study involved a study group of 12 persons who used snuff and had leukoplakia in the mucosa of the oral cavity and a control group of 12 healthy persons who did not use tobacco and did not have leukoplakia of the oral cavity. A 5-mm punch biopsy was obtained from the center of an area of leukoplakia and from a corresponding normal-appearing area of buccal mucosa from each of the snuff-using individuals. In the same manner, one biopsy was obtained from the buccal mucosa from each of the 12 nontobacco-using persons. Consent for biopsy was obtained from all study participants according to the guidelines of the Institutional Review Board of the University Hospital.

All biopsy specimens were bisected immediately; one half was preserved in 10% formalin and later embedded in paraffin, and the other half was quick-frozen in liquid nitrogen and stored at −80° F. Both paraffin-embedded and frozen specimens were processed immunohistochemically.

The second portion of the study involved 43 paraffin-embedded leukoplakia specimens collected between 1985 and 1992, available in the pathology archives of the University Hospital, Oklahoma City, Okla. The degree of epithelial dysplasia present in each specimen was graded by a pathologist, using light microscopy examination of hematoxylin and eosin–stained sections, according to the following criteria: *mild dysplasia,* squamous cell hyperplasia, hyperkeratosis with signs of dysplasia; *moderate dysplasia,* no exact criteria exist, but cellular and structural abnormalities are intermediate between those present in mild and severe dysplasia; and *severe dysplasia,* pronounced cellular atypia with many irregular and hyperchromatic nuclei, significant loss of basal cell polarity, but with identifiable squamous differentiation.

Tissue used for positive controls was donated by Dr. William Bennett (National Cancer Institute, Bethesda, Md.; human squamous cell carcinomas implanted into nude mice) and Dr. J. P. Klein-Szanto (Fox Chase Cancer Center, Philadelphia, Pa.; human lung carcinomas). Positive cancer controls were also identified by immunohistochemical analysis of specimens from the archives of the Department of Pathology at the University of Oklahoma, Health Sciences Center. These positive cancer controls were used with each batch of specimens to confirm reliability of the technique.

Immunohistochemical Processing

Four primary antibodies (PAs) were chosen to maximize the possibility of identifying accumulation of p53 protein: PAb 421[21] and PAb 1801[22] (Oncogene Science, Inc., Uniondale, N.Y.), which are murine monoclonal antibodies to human p53 protein that recognize specific epitopes of both the wild-type and mutated forms of p53 protein; PAb CM-1[23] (Signet Laboratories, Dedham, Mass.), which is a polyclonal rabbit antibody raised against both wild-type and mutant human p53 protein; and PAb 240[24] (Oncogene Science, Inc.), which recognizes only mutated forms of p53 protein. Thus it would be expected that three of the four antibodies would recognize wild and/or mutant forms of p53, whereas one (PAb 240) recognizes only mutant forms.

Six-micrometer sections were cut with a cryostat and mounted on glass slides (Probe-On Plus, Fisher Scientific Co., Pittsburgh, Pa.). Frozen sections were allowed to air dry for 5 to 10 minutes and then were

fixed in acetone for 15 minutes at 4° C, rinsed in phosphate-buffered saline (PBS), air dried, and stored at −20° C. Before processing, frozen sections were allowed to come to room temperature.

Six-micrometer sections were also cut from the paraffin-embedded specimens. These sections were mounted on glass slides and incubated at 40° C for 45 minutes to assist with deparaffinization. They were then taken through two washes of xylene (of 10 minutes each) and alcohol washes graded down to distilled water.

All prepared slides were then rinsed three times in PBS and 0.05% Tween-20. To block endogenous peroxidase activity, we immersed the slides in hydrogen peroxide (0.6%) for 15 minutes at room temperature. For reduction of nonspecific background staining, the sections were incubated with horse immunoglobulin G for the monoclonal antibodies or goat immunoglobulin G for the polyclonal antibody, for 30 minutes at room temperature. The sections were then incubated with each PAb (1:50 dilution) for 1 hour at 45° C. Sections to be used as negative controls were incubated with the vehicle solution for the primary antibody, and the PAb was left out of the solution. Sections were then washed in PBS three times and were incubated with biotinylated secondary antibody (Vectastain Elite Kit; Vector Laboratories, Burlingame, Calif.) at 1:200 dilution for 45 minutes. After three rinses in PBS, the sections were incubated with the avidin-biotin complex (Vector Laboratories) at 1:200 dilution, followed with 3,3′-diaminobenzidine 0.05 mg/ml hydrogen peroxide for 5 minutes, and rinsed twice with PBS.

The staining process was standardized by use of an automated processor, the Histomatic Code-On processor (Fisher Scientific, Inc.). This was done to reduce variability in the staining protocol because all specimens for one batch were taken through each step at exactly the same time and under the same conditions.

The sections were counter-stained with hematoxylin, dehydrated through graded alcohols, cleared, and then cover-slipped with DPX mounting medium (BDH Laboratory Supplies, Poole, England). The slides were examined with light microscopy (Olympus Vanox AH-2 fitted with differential interference control optics; Olympus Corp., Lake Success, N.Y.).

To confirm the specificity of positive staining, we performed repeat analysis with serial dilutions of primary antibody with selected positive specimens. PAb 421 and PAb CM1 were diluted to 1:25, 1:50, and 1:100, and PAb 1801 was diluted to 1:50, 1:100, and 1:200. Selected positive specimens showed a primary antibody concentration dependency (i.e., serial increases in primary antibody concentration resulted in sequentially more intense nuclear staining). The 1:50 dilution was selected for analysis of specimens because it produced the most distinct nuclear staining.

To control for background staining, we incubated identical serial sections with the PAb vehicle (PBS + 20% bovine serum albumin + 0.05% Tween-20) without the primary antibody.

Data Analysis

Determination of positive staining was made on the basis of intense full nuclear staining with a brown coloration. The number of cells stained in five adjacent high-power fields and the location of cells relative to the basal cell layer were recorded. The staining observed with antibodies 1801 and CM1 complemented each other and produced the most consistent staining patterns. These two antibodies were therefore used to quantify the number of cells staining positive. The cell counts reported were the average of these two antibodies.

Statistical analysis was performed on a personal computer equipped with Statistica software (Statsoft, Tulsa, Okla.). Differences between groups were analyzed with a one-way analysis of variance, and correlation of coefficients were calculated by use of the Spearman's rank order correlation test for nonparametric data. The alpha level was set at 0.05.

RESULTS

The positive control specimens consistently stained positive with all four antibodies.

Part 1

Histologic examination of the leukoplakia specimens from snuff-users demonstrated mild epithelial dysplasia in all 12 cases. The epithelium of the normal-appearing mucosa from the persons who used snuff and from the nontobacco-using persons was found to be histologically normal. Accumulation of p53 protein was detected in 5 of the 12 (41.7%) leukoplakia specimens. The average of the total number of positive cells in the leukoplakia of snuff-users (21.89 ± 4.33; mean ± SE) was higher than that of normal-appearing mucosa (4.00 ± 1.0) and that of nontobacco-using controls (7.00 ± 5.04) (Fig. 1). The difference between the number of positive cells in the leukoplakia specimens and that of the normal tissue from the same persons was

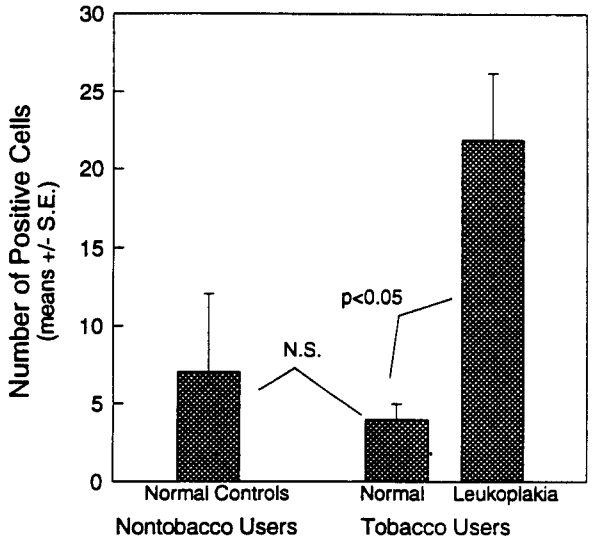

Fig. 1. Means of number of positive cells in five consecutive high-power fields from leukoplakia and normal tissue. *NS*, Not significant.

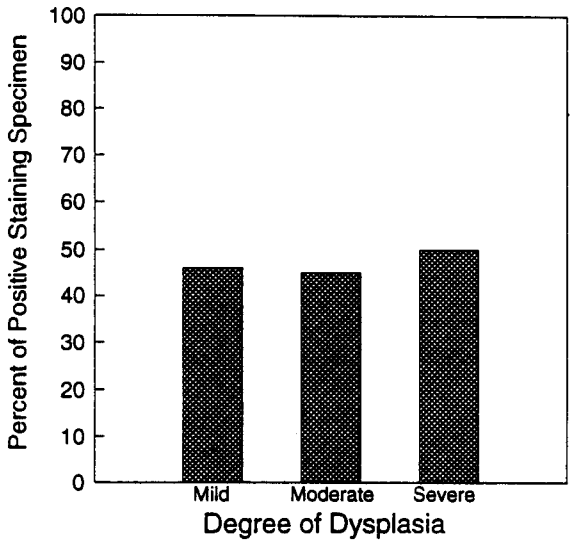

Fig. 2. Percent of specimens with any positive-staining cells.

statistically significant ($p < 0.05$). The difference between the number of positive cells in the leukoplakia specimens and that of the controls was not significant ($p = 0.06$). The difference between the number of positive cells in normal-appearing mucosa of snuff-users and the normal mucosa of nontobacco-using controls was not statistically significant ($p > 0.05$).

Part 2

There were 24 specimens with mild dysplasia, 11 specimens with moderate dysplasia, and 8 with severe dysplasia. The proportion of specimens that contained at least one cell staining positive was about 50% in all histologic categories of dysplasia (Fig. 2). However, the number of positive-staining cells correlated with the degree of dysplasia ($r = 0.853$) (Fig. 3). In fact, the average number of positive cells in the specimens showing moderate dysplasia (140.36 ± 30.03) and severe dysplasia (232.86 ± 26.85) was significantly higher than that in the specimens showing mild dysplasia (14.53 ± 3.33) ($p < 0.05$) (Fig. 4).

In controls that were processed with no primary antibody, no staining was noted in the paraffin section preparations; however, frozen specimens did demonstrate reproducible background cytoplasmic staining thought to be caused by endogenous peroxidase activity that did not block with our low-concentration hydrogen peroxide blocking step. In the positive cancer controls, this background staining was consistently located within tissue macrophages.

DISCUSSION

The results of this study indicate that accumulation of the p53 protein may be a useful biomarker in monitoring leukoplakia of the oral cavity in snuff-users, with regard to the potential progression of this lesion to squamous cell carcinoma.

In the leukoplakia of the snuff-users we studied, the average number of positive-staining cells was significantly higher than in the normal-appearing epithelium of the same persons; however, the proportion of specimens that contained at least one positive staining cell was only 41.7%. This is not unexpected and is probably because the leukoplakia specimens studied were very homogeneous in their histology, with only mild epithelial dysplasia present. Previous investigations have shown that accumulation of p53 occurs in only a small proportion of other epithelial lesions with mild dysplasia.

If we accept progression in the severity of dysplasia as a reflection of the transition from oral leukoplakia to carcinoma, the results of the second part of our study suggest that accumulation of the p53 protein is an early event in the oncogenic process related to oral leukoplakia. The parallel increase in the number of p53-positive cells and the degree of dysplasia that we observed in this study has also been observed by Bennett et al.[25] in the epithelium surrounding esophageal carcinomas. Interestingly,

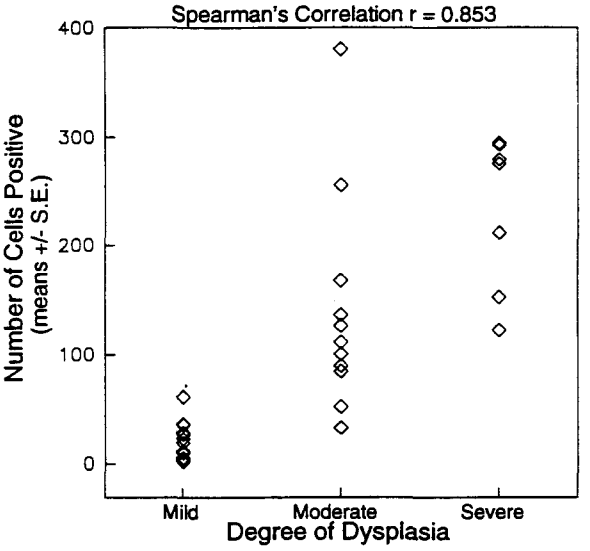

Fig. 3. Correlation analysis between degree of dysplasia and number of positive-staining cells in any positively staining specimen.

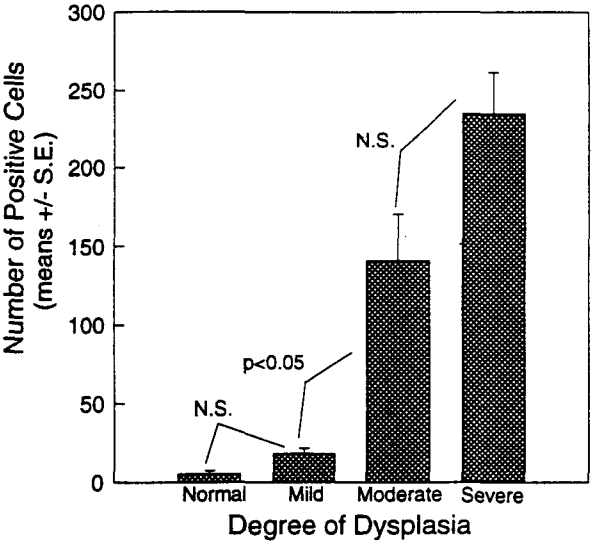

Fig. 4. Means of number of positive cells in five consecutive high-power fields from leukoplakia specimens with various degrees of epithelial dysplasia and from normal tissue. *NS*, Not significant.

these authors observed one example of esophageal dysplasia in which p53 mutation and protein accumulation occurred before invasion developed. A similar correlation between the degree of dysplasia and the accumulation of p53 protein has been demonstrated in colon adenomas.[20] In this regard, Pignatelli et al.[16] have speculated that because p53 protein accumulation does not correlated with established prognostic indicators for colorectal carcinoma, the genetic alteration seen in these early premalignant lesions, such as adenomas of the colon, may be involved in the early steps of the adenoma/carcinoma oncogenic process. A similar role for alterations in the p53 gene could be operative in oral leukoplakia. It is therefore reasonable to expect that cessation of snuff use or interventions that may halt the oncogenic process in oral leukoplakia will be evidenced by corresponding changes in the accumulation of p53 protein.

The observed lack of significance in the difference between the accumulation of p53 protein in the leukoplakia specimens and the nonsmoking control specimens may be a result of the small number of persons studied, particularly because the difference in the number of positive cells is small. It is also possible that the use of snuff initially decreases the accumulation of p53 protein in the oral epithelium, before dysplasia becomes histologically apparent. The latter explanation is less likely. Shea et al.,[26] studying p53 protein accumulation in basal cell carcinomas of the skin, observed significantly less accumulation of this protein in the epithelium of the surrounding normal-appearing skin, and no accumulation in control samples of skin chronically protected from sunlight. These authors hypothesized that the p53 protein accumulation in the exposed, but normal, epithelium may represent cellular attempts to prevent genomic incorporation of mutations. We were not able to demonstrate a similar "field effect" in the normal-appearing oral mucosa of snuff-users. Such a field effect, if present in persons with oral leukoplakia, may be influenced by duration and extent of tobacco use, as well as other variables not taken into account by this investigation.[27,28]

It is not possible to ascertain that the p53 protein detected in the leukoplakia specimens in this investigation is actually the result of mutation. In fact, none of the leukoplakia specimens stained positive with antibody 240, which recognizes only mutated p53 protein. Whereas the presence of staining with PAb 240 would have been definitive evidence for the presence of mutated p53, the absence of staining does not exclude the presence of mutated forms not recognized by this antibody. In addition, PAb 240 may not have bound well for formalin-fixed specimens because we observed that it also gave poor results with cancer controls. Realizing the chronic nature of snuff-induced oral leukoplakia, its ability to regress when snuff use is discontinued, and the long period over which cancer usually develops, it is

possible that the positive p53 staining demonstrated in some of our specimens does not represent mutation, but represents an induction of wild-type p53 production and/or posttranscriptional stabilization of the protein. This hypothesis will have to be examined with DNA analysis to identify mutations within the p53 gene. Using special flow cytometry techniques, Kastan et al.[29] have been able to demonstrate increased levels of wild-type p53 protein in human hemopoietic cells after radiation-induced DNA damage. They propose that this may be caused by a posttranscriptional mechanism that stabilizes p53 protein and prolongs its half-life. Thus p53 may inhibit the mitotic cycle while DNA repair mechanisms prevent the permanent incorporation of mutations into the DNA.[30,31]

In summary, we have demonstrated p53 protein accumulation in snuff-related oral leukoplakia specimens to be significantly greater than that seen in the normal tissue from the same person. We have also demonstrated that p53 protein accumulation in oral leukoplakia is correlated with the degree of epithelial dysplasia present in the specimen. Consequently, the accumulation of p53 protein may be a biomarker useful for monitoring the effect of prevention interventions in persons with oral leukoplakia.

REFERENCES

1. McGuirt WF. Snuff dipper's carcinoma. Arch Otolaryngol Head Neck Surg 1983;109:757-60.
2. Poulson TC, Lindenmuth JE, Greer RO. A comparison of the use of smokeless tobacco in rural and urban teenagers. CA Cancer J Clin 1984;34:248-61.
3. Greer RO, Poulson TC. Oral tissue alterations associated with the use of smokeless tobacco by teen-agers. Oral Surg 1983;56:275-84.
4. Friedell HL, Rosenthal LM. Etiologic role of chewing tobacco in cancer of the mouth. JAMA 1941;116:2130-4.
5. Winn DM, Blot WJ, Shy CM, et al. Snuff dipping and oral cancer among women in the southern United States. N Engl J Med 1981;304:745-9.
6. Jayant K, Balakrishnan V, Sanghv LD, Jussawalla DJ. Quantification of the role of smoking and chewing tobacco in oral, pharyngeal and oesophageal cancers. Br J Cancer 1977;35: 232-5.
7. Silverman S, Gorsky M, Lozada G. Oral leukoplakia and malignant transformation: a follow-up study of 275 patients. Cancer 1984;53:563-8.
8. Kramer IRH, Lucas RB, Pindborg JJ, Sobin LH. Definition of leukoplakia and related lesions: an aid to studies on oral precancer. (WHO Collaborating Center for Oral Precancerous Lesions.) Oral Surg 1978;46:518-39.
9. Lind PO. Malignant transformation oral leukoplakia. Scand J Dent Res 1987;95:449-55.
10. Heyne KE, Lippman SM, Hong WK. Chemoprevention in head and neck cancer. Hematol Oncol North Am 1991;5: 783-95.
11. Gilchrist JM, Thompson GC, Medina JE. Markers of keratinocyte differentiation in snuff-induced leukoplakia. Am J Surg 1992;164:563-6.
12. Lippman SM, Lee JS, Lotan R, et al. Biomarkers as intermediate endpoints in chemoprevention trials. J Natl Cancer Inst 1990;82:555-60.
13. Finlay CA, Hinds PW, Levine AJ. The p53 proto-oncogene can act as a suppressor of transformation. Cell 1989;57:1083-93.
14. Hollstein M, Sidransky D, Vogelstein B, Harris CC. p53 Mutations in human cancers. Science 1991;253:49-53.
15. Bartck J, Bartkova J, Vojtesek B, et al. Patterns of expression of the p-53 tumor suppressor gene in human breast tissues and tumours in situ and in vitro. Int J Cancer 1990;46:839-44.
16. Pignatelli M, Stanp GWH, Kafiri G, et al. Over-expression of p53 nuclear oncoprotein in colorectal adenomas. Int J Cancer 1992;50:683-8.
17. Ramel S, Reid BJ, Sanchez CA, et al. Evaluation of p53 protein expression in Barrett's esophagus by two-parameter flow cytometry. Gastroenterology 1992;102:1220-8.
18. Dolcetti R, Doglioni C, Maestro R, et al. p53 Over-expression in an early event in the development of human squamous-cell carcinoma of the larynx: genetic and prognostic implications. Int J Cancer 1992;52:178-82.
19. Kikuchi-Yanoshita R, Konishi M, Ito S, et al. Genetic changes of both p53 alleles associated with the conversion from colorectal adenoma to early carcinoma in familial adenomatous polyposis and non-familial adenomatous polyposis patients. Cancer Res 1992;52:3965-71.
20. Langdon JD, Partridge M. Expression of the tumour suppressor gene p53 in oral cancer. Br J Oral Maxillofac Surg 1992;30:214-20.
21. Lane DP, Crawford LV. T antigen is bound to a host protein in SV40-transformed cells. Nature 1979;278:261-3.
22. Banks L, Matlashewski G, Crawford L. Isolation of human p-53-specific monoclonal antibodies and their use in the studies of human p53 expression. Eur J Biochem 1986;59: 529-34.
23. Midgley CA. Analysis of p53 expression in human tissues. J Cell Sci 1992;101:183-9.
24. Gannon JV, Greaves R, Iggo R, Lane DP. Activating mutations in p53 produce a common conformational effect. A monoclonal antibody specific for the mutant form. EMBO J 1990;9:1595-602.
25. Bennett WP, Hollstein MC, Metcalf RA, et al. p53 Mutation and protein accumulation during multistage human esophageal carcinogenesis. Cancer Res 1992;52:6092-7.
26. Shea CR, McNutt NS, Volkenandt M, et al. Over expression of p53 protein in basal cell carcinomas of human skin. Am J Pathol 1992;141:25-8.
27. Grady D, Greene J, Daniels TE, et al. Oral mucosal lesions found in smokeless tobacco users. JADA 1990;121: 117-23.
28. Ernster VL, Grady DG, Greene JC, et al. Smokeless tobacco use and health effects among baseball players. JAMA 1990; 264:218-24.
29. Kastan MB, Onyekwere O, Sidransky D, et al. Participation of p53 protein in the cellular response to DNA damage. Cancer Res 1991;51:6304-11.
30. Vogelstein B, Kinzler KW. p53 Function and dysfunction. Cell 1992;70:523-6.
31. Hall PA, McKee PH, Menage HDP, et al. High levels of p53 protein in UV-irradiated normal human skin. Oncogene 1993;8:203-7.