Aberrations of the Tumor Suppressor p53 Gene and p53 Protein in Solar Keratosis in Human Skin

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Aberrations of the p53 gene in 27 solar keratoses were examined by the polymerase chain reaction and single-strand conformation polymorphism and DNA sequencing analyses. In a series of Japanese patients, eight of 27 cases (30%) of solar keratosis showed structural abnormalities of the p53 gene. Six of eight aberrations of p53 gene were determined to be single nucleotide substitutions, and five of these were located at a dipyrimidine site. In solar keratosis, noticeable nucleotide substitutions, and especially a double-base change, such as specific to ultraviolet (UV) light [5-8]. SCC may develop from solar keratosis (SK), which is a result of long-term exposure to sunlight.

The abnormal accumulation of p53 protein was found by the immunohistochemical staining with antibody to p53 protein in the nuclei of SK [9-11]. Immunohistochemical detection of p53 protein, however, may not indicate the presence of p53 gene mutations [4]. To clarify the presence of p53 gene alteration in SK, we used a combination of the polymerase chain reaction and single-strand conformation polymorphism (PCR-SSCP) analysis [12] and direct DNA sequencing analysis. A correlation between alteration of the p53 gene and accumulation of p53 protein was also analyzed.

MATERIALS AND METHODS

Sample
Fresh specimens of 27 SK were excised from 25 patients between June 1991 and December 1992 under the collaboration of Department of Dermatology of the National Cancer Center Hospital; Saitama Medical School; School of Medicine, Yokohama City University; School of Medicine, Kyushu University; and the School of Medicine, Kagoshima University. Surgical specimens were either fixed with the Amex (acetone-methyl benzoate-xylene and paraffin embedding) method [13] of embedded in OCT compound (Tissue-Tek, Miles) to perform tissue sections, and were subsequently sent to the Epidemiology Division, National Cancer Center Research Institute, and stored at -80°C until use. Genomic DNA extractions were performed on Amex sections and frozen sections (50 μm in thickness) using the proteinase K-phenol-chloroform extraction method as described by Gooley et al [14]. Five normal parts of the biopsied skin with SK and five skin tissues from the non-sun-exposed areas were used for control specimens.

PCR-SSCP Analysis
DNA samples (0.1 μg) were subjected to a polymerase chain reaction (PCR) using two appropriate oligonucleotides as primers [15]. The oligonucleotide primers for PCR amplification of all p53 exons (exon 2 to 11) were designed as described previously [16]. The PCR was carried out in 17 μl solution containing 1.25 mM dATP, 1.25 mM dGTP, 1.25 mM dCTP, 1.25 mM dTTP, 0.75-1.0 mM MgCl2, 10 μM primers, 0.1 μg of sample DNA, 0.2 μl of [α-32P] dCTP (3000 μCi/mmole, 10 mCi/μl, Amersham), 0.5 unit of Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT) in 10 mM Tris-HCl, pH 8.3, containing 0.1% gelatin, and 12 μl mineral oil. The samples were subjected to 30 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 60 seconds, on a DNA thermal cycler (Perkin-Elmer Cetus). On another occasion, the 5'-ends of these primers were labeled by the polymerase kinase reaction with [γ-32P]ATP as described previously [17].

The PCR mixture (5 μl) was heated at 80°C with 45 μl of formamide dye mixture (95% formamide dye, 20 mM ethylenediaminetetraacetic acid, 0.05% xylene cyanol, 0.05% bromphenol blue), and then 1 μl of the preparation was applied to 5% polyacrylamide gel containing 45 mM Tris-borate (pH 8.3) and 4 mM ethylenediaminetetraacetic acid. Glycerol (5%) was added for better separation of bands. Electrophoresis was performed at 30 W for 2 to 4 h with cooling by a fan. The gel was dried on filter paper and exposed to X-ray film at -80°C for 4 to 12 h using an intensifying screen. The PCR-SSCP analysis was repeated 2-4 times to confirm the same mobility shift pattern.

Direct DNA Sequencing
DNA fragments showing repeatedly the same mobility shift pattern by PCR-SSCP analysis were eluted from polyacrylamide gel as described previously [18], and were amplified by 30 cycles of the symmetric PCR under the same conditions as described earlier. The reaction mixture was diluted and deionized in a Centricon 30 microconcentrator (Amicon, Danvers, MA). After the amplified DNAs had been annealed with the 5' labeled primers, nucleotide sequences were determined using a double-strand DNA Cycle Sequencing System (Gibco BRL).

Immunohistochemistry
Immunohistochemical staining was performed on Amex sections and frozen sections using an avidin-biotin-peroxidase

Manuscript received February 4, 1994; accepted for publication May 13, 1994.

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Abbreviations: Amex, acetone-methyl benzoate-xylene and paraffin embedding; PCR-SSCP, polymerase chain reaction and single-strand conformation polymorphism; SK, solar keratosis.
complex method as described by Hiyoshi et al [19]. The monoclonal antibodies to p53 protein, pAb 1801, and DO7 were obtained from Oncogene Science Inc. and Novocastra Laboratories Ltd., respectively. As positive controls, we used AMEX-fixed colon cancer tissue sections. Negative controls were tissue sections that were incubated without the primary antibody.

RESULTS

Aberrations of the p53 Gene in SK

The number of cases showing the immunohistochemical accumulation of the p53 protein in the nuclei and/or an aberration of the p53 gene by PCR-SSCP analysis was 10 of 27 SK (Table I and Fig 1a, b). In PCR-SSCP analysis, DNA extracted from non-sun-exposed normal skin (five normal part of biopsied SK and five from non-sun-exposed area) did not show any abnormal band mobility. An aberration of the p53 gene was present in eight of 27 SK (30%). Histologically, the Bowenoid type was found to have mutations in a higher proportion of the cases, six of 9 (67%), and the atrophic type was found less frequently, in two of 11 cases (18%). Mutation was not detected in the five cases with a hypertrophic type lesion in the two cases with an acantholytic type lesion. The mutations were detected in exons 4, 6, 7, 8, and 10. Six of eight mutations were single-nucleotide missense mutations (Fig 2a). Cases SK4, SK27, and SK33 showed the p53 gene alteration, which lost the p53 protein production because of nonsense mutation to stop codon, or by several base deletion (Fig 2b and Table I). Base substitutions were three C to T and one T to C transitions, and one each T to G and C to A transversions. Dipyrimidine sites were involved in five cases. Three C to T substitutions occurred on the CpG methylation site (Table I).

Immunohistochemical Analysis of the p53 Protein

p53 protein was stained in the nuclei of six of 27 SK (Table I and Fig 3). Degree of positive nuclear staining varied by cases, but mostly more than half nuclei were stained. It was not detected in the neighboring normal epidermal cells and stromal cells.

Relationship Between p53 Gene Mutations and Nuclear Accumulation of p53 Protein

The nuclear staining for p53 protein and the presence of the p53 gene alterations were not statistically associated (p < 0.08, Fisher’s exact test). Association between the above two, however, became significant when only the missense mutation was accounted (p < 0.01, Fisher’s exact test) (Table II). In SK25, nuclear staining of the p53 protein was negative, although the mutation from T to G was detected. This mutation was different from the common UV-specific substitution at dipyrimidine site. The other three cases (SK33, SK27, and SK4) that were negative for nuclear staining had genetic changes to stop codon type (Table I). In SK5 and SK40, aberrations of the p53 gene were not detected despite the positive nuclear staining of p53 protein (Table I).

DISCUSSION

We formerly reported the usefulness of immunohistochemical staining with anti-p53 protein antibody [11]. An accumulation of the p53 protein in the nuclei usually suggested the presence of a mutation in the gene, although p53 accumulation was also observed shortly after UVB irradiation [20]. Campbell et al [21] reported that p53 protein accumulated in the normal human skin as early as 24 h after UVB or UVC irradiation. In acute irradiation, p53 protein may accumulate by direct binding to the damaged DNA or as a result of binding to heat-shock protein [22]. The gene sequence analysis in this study revealed that two cases had no mutation at all in the p53 exons regardless of the positive p53 staining.

Four of six cases (67%) showed a significant coincidence between positive staining of p53 protein and gene alteration. UV light may produce specific mutations, leaving a “signature” in the DNA. Mutations due to direct absorption of UV light by DNA are predominantly C to T transitions at dipyrimidine sites, including CC to TT.
double-base mutations [5–8]. The appearance of C to T transitions exclusively at dihydrimidine sites is also frequent by UV exposure. Chance would dictate that one in four would occur at a monohydrimidine site [1]. Frequent C to T pattern of mutation was also reported by Campbell et al [23] in Bowen’s disease. They suggested that the initial mutagenic lesion in the p53 gene in these lesions was dihydrimidine photoproduts induced by UV irradiation [23].

The spectrum of observed mutations in the case of solar keratosis was different from those observed by Brash et al [1] in SCC from sun-exposed skin. We found that five of eight cases (62.5%) of SK contained a mutation spectrum at dihydrimidine site specific for UV. Other uncommon UV specific mutations were one each of C to A and T to C nucleotide changes. These changes, however, were also found in the experimental UV irradiation [7,8]. At least CC or GG sequence was involved in the two short deletion sites in our study (Table I). Molés et al reported the similar change in BCC [4]. Methylation of CpGs in normal tissues may increase the probability of mutations at such sites because of the ability of 5-methylcytosine to undergo deamination and result in thymine [24]. The p53 gene mutation in BCC at CG dinucleotides suggests that the particular mutation may be due to the 10^6-fold acceleration of cytosine deamination rate by cyclobutane dimers, occurring at 5-methylcytosine [25]. Nearly one third of the 280 human p53 tumor mutations were transitions at hot spor codons with CpG sites (codons 175, 196, 213, 248, 273, and 282) in human cancer of internal organs [26]. In recent studies, mutations at codon 248 were frequently observed in BCC and SCC by UV exposure [1,3,25]. In our series, one of eight (12.5%) SK cases showed transitions at hot spot codon 248 in p53 gene. Four cases were negative for p53 immunohistochemical staining, although p53 gene aberrations were present. These were attributed to nonsense mutation to the stop codon or several base deletions. Ziegler et al [25] reported that the arginine changed to a stop codon (codon 342) in p53 gene in BCC. In the arginine codon being CGA in the p53 gene, these changes are likely to appear because of the ability of 5-methylcytosine to undergo deamination.

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The use of immunohistochemical staining is a convenient and a useful tool for screening the p53 missense mutations. Combination of both immunohistochemical staining and PCR-SSCP analysis, however, is necessary because the non-production of p53 protein due to gene mutation to stop codon was not negligible in p53 protein negative cases.

In the study of p53 knockout mice, Kemp et al [28] reported that lack of p53 not only increases the rate of benign to malignant conversion, but also seems to drive further changes toward loss of differentiation with increasing malignancy. Furthermore, they investigated that other genetic alterations, which may be facilitated by mutant ras acting synergistically with lack of p53 gene, were responsible for driving malignant progression [28]. In our study, SK having alteration of p53 gene may possess ability to transform into SCC. In our preliminary work, by using the same SK sample in this study, we found a point mutation of Ha-ras without any p53 mutation in one case (unpublished data).

A follow-up study of SK patients with p53 alteration is necessary.

**Table II. Association of p53 Gene Missense Mutation with Nuclear Staining of p53 Protein in Solar Keratosis**

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<tr>
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<th>With p53 Gene Mutation</th>
<th>Without p53 Gene Mutation</th>
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<tr>
<td>Nuclear staining</td>
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<tr>
<td>Positive</td>
<td>4 (67%)</td>
<td>2 (33%)</td>
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<tr>
<td>Negative</td>
<td>1 (5%)</td>
<td>17 (94%)</td>
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p < 0.01
to confirm the relationship between the p53 alterations in premalignant skin lesions and the progression to SCC.

We appreciate Dr. C.C. Harris, Division of Cancer Etiology, National Cancer Institute, for his constructive discussion. A part of this work was supported by the research fund for the Effects of Ultraviolet Ray Increase on Human Health from the Global Environment Research, Environmental Agency, and Grant-in-Aid for Cancer Research supported by the Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health and Welfare.

REFERENCES


