Frequent p53 Accumulation in the Chronically Sun-Exposed Epidermis and Clonal Expansion of p53 Mutant Cells in the Epidermis Adjacent to Basal Cell Carcinoma

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p53 expression was studied immunohistochemically to identify a precursor lesion of basal cell carcinoma (BCC) in the epidermis adjacent to BCC. With two different anti-p53 antibodies of CM1 and DO7, p53 expression was frequently detected in the epidermis adjacent to BCCs arising on the face and in the normal epidermis with usual sun exposure. In the epidermis adjacent to BCC, stained cells were occasionally clustered in a small area, but no cluster was found in the normal epidermis with usual sun exposure. The expression was less frequent in the normal epidermis with rare sun exposure. Ten cases of normal skin with usual sun exposure, showing CM1 staining in the epidermis, were screened for p53 gene mutations with polymerase chain reaction–single-strand conformation polymorphism analysis using DNAs obtained from the epidermis. No mutation was detected in exons 2 to 10 of the p53 gene in these 10 cases. The epidermis flanking three BCCs that was stained with CM1, on the other hand, carried a missense mutation of C to G transversion at a dipyrimidine site of codon 249. This alteration replaced arginine with threonine. The mutation of codon 249 was not detected in the three BCCs. Our results first suggest that ultraviolet light irradiating the skin in a daily life induces p53 accumulation in the epidermis and secondly that the frequent clonal expansion of p53 mutant cells occurs in the epidermis adjacent to BCCs. This clonal expansion of mutant p53 may provide a molecular basis for high risk of developing subsequent new skin cancers in patients with BCC.

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It is widely accepted that tumorigenesis is a multistep process that involves activation of protooncogenes and inactivation of tumor-suppressor genes [1,2]. This process has been best understood in colorectal cancer in which the histopathologic progression is often correlated with specific genetic changes [3]. Alterations of the p53 tumor-suppressor gene, which are the most common genetic changes in various types of tumors [4], frequently occur at a late stage in colorectal carcinogenesis. Mutations in the p53 gene have also been often detected in basal cell carcinoma (BCC) of the skin [5–10]. Epidemiologic observations that ultraviolet (UV) light is involved in carcinogenesis of BCC [11] have been supported at a molecular level by predominant mutations of C to T transitions at dipyrimidine sites, including CC to TT substitutions, in BCC. Although relevance of p53 gene mutations to carcinogenesis of BCC has been indicated, whether p53 mutations occur as an early event or a late event of the carcinogenesis remains to be answered. It may be difficult to clarify this, because the majority of BCCs do not arise from precursor lesions but rather appear *de novo* [12].

Immunohistochemical detection of p53 proteins has become a common approach to assess potential p53 mutations in tumors [13]. The accumulation of p53 has been thought to be mainly caused by elongated half-lives of the proteins caused by mutations [14], although recent evidence suggests that the correlation between immunohistochemical overexpression and underlying mutations is not absolute [9,10,15] and that there are other mechanisms for the accumulation by DNA damaging agents including UV light [16–18]. Immunohistochemical overexpression of p53 has been observed not only in invasive tumors but also in precancerous or early lesions of tumors of the skin [19–21]. Overexpression of p53 might be a useful marker to identify precursor lesions of cancers arising *de novo*, which are histopathologically indistinguishable from normal cells. In the present study, p53 expression in the epidermis adjacent to BCC was immunohistochemically examined because precursor lesions of BCC might be identified by p53 staining. In samples showing immunostaining in the epidermis, p53 gene mutations were screened by polymerase chain reaction–single-strand conformation polymorphism (PCR-SSCP) analysis using epidermal DNAs obtained from microdissected tissue materials. Mutated sequences were confirmed by DNA sequencing. Our results indicated clonal expansion of p53 mutant cells in the epidermis adjacent to BCC,

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Abbreviation: SSCP, single-strand conformation polymorphism.

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Table I. Immunohistochemical p53 Expression in Normal Skin and Basal Cell Carcinoma

Specimens	Age Mean (range)	Sex (F/M)	Site Examined	Number of Specimens Examined	Number of Specimens Stained with CM1 (%)	p[a] Value	DO7 (%)	p[a] Value
Normal epidermis			Face	11	8 (73)		5 (45)	
			Scalp	3	2 (67)		2 (67)	
			Ear	2	1 (50)		0 (0)	
Usual sun exposure	41 (1–67)	4/12	Total	16	11 (69)	<0.05	7 (44)	<0.05
			Trunk	8	1 (13)		0 (0)	
			Buttock	2	1 (50)		0 (0)	
Rare sun exposure	47 (3–87)	7/4	Sole	1	0 (0)		0 (0)	
			Total	11	2 (12)		0 (0)	
Basal cell carcinoma								
Tumor	69 (51–91)	11/6	Face	17	15 (88)		11 (65)	
Adjacent epidermis	69 (51–91)	11/6	Face	17	11 (65)	<0.05	7 (41)	<0.05

[a] The frequency showing immunohistochemical staining in the normal epidermis with usual sun exposure and that in the epidermis adjacent to BCC were statistically compared with that in the epidermis with rare sun exposure using the chi-square test. Values used for the comparison are frequencies in total cases.

providing a possible molecular basis for the high risk for subsequent skin cancers in patients with BCC.

MATERIALS AND METHODS

Specimens A total of 44 specimens (8 mm in diameter) were obtained from 43 Japanese patients. Details of specimens are summarized in **Table I**. Seventeen samples of BCC consisted of 13 of a solid type and four of an adenoid type. No BCC patients had received chemotherapy. Twenty-seven samples of the normal skin were obtained from 26 patients undergoing plastic surgery. The specimens were quickly frozen in liquid nitrogen and stored at −70°C until use.

Immunohistochemistry A mouse monoclonal anti-p53 antibody of DO7 (Novocastra Laboratory, Newcastle-upon-Tyne, UK), and a rabbit polyclonal anti-p53 antiserum of CM1 (Novocastra Laboratory) were used as primary antibodies to detect p53. DO7 and CM1 were applied at dilutions of 1:100 and 1:1500 as recommended by the supplier, respectively. Frozen 6-μm–thick sections were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 30 min at 4°C. Fixed sections were immunostained by an avidin-biotin-peroxidase complex method as previously described [22]. The sections were counterstained with methyl green. As positive controls for the immunostaining, we used a SV40-transformed human lung fibroblast cell line, W1-38 VA13 sub2 RA, supplied by Japanese Cancer Research Resources Bank, and a human cutaneous squamous cell carcinoma cell line established in this laboratory, which abundantly expressed p53 (data not shown). As negative controls for the immunostaining, we used normal mouse IgG (10 μg/ml) instead of DO7, and normal rabbit serum (1:1500 dilution) instead of CM1. In comparison with negative controls, clearly stained nuclei were regarded as specific staining. Cases showing specific staining were counted as positive even though stained nuclei were small in number. Specific staining was judged independently by three observers (*TA*, *SK*, and SS) without prior knowledge of the clinical data. Statistical analyses were carried out using the chi-square test.

DNA Preparation DNA was prepared as previously described [9]. Briefly, DNAs of the epidermis were prepared from five frozen 6-μm–thick sections on microscopic glass slides, which were consecutive to those used for immunohistochemical staining. To enrich DNAs of the epidermis showing p53 overexpression, epidermal portions corresponding to those showing immunohistochemical p53 staining were microdissected with a needle under microscopic observation. DNAs of BCC were prepared from portions of tumor nests more than 2 mm apart from the epidermis to avoid cross-contamination. To avoid contamination, this step was performed carefully by using a new needle in each section. DNAs of peripheral blood cells from patients were used as those of normal tissue.

PCR-SSCP Analysis and DNA Sequencing Mutations in exons 2 to 10 of the p53 gene were first screened by PCR-SSCP analysis as previously described [9]. Oligonucleotide primers to amplify exons 2 to 10 are listed in **Table II**. PCR-SSCP was repeated at least twice in each case showing a mobility shift to ensure that the results were reproducible. Bands with altered migration in SSCP analyses were extracted from a gel, re-amplified by PCR and cloned into the pCR II vector using a TA cloning kit (Invitrogen Co., San Diego, CA). Cloned DNAs were sequenced by fluorescence-based dideoxy-termination methods using AmpliTaq DNA polymerase and an Applied Biosystems Model 373A automated DNA sequencer (Perkin-Elmer/Division of Applied Biosystems, Foster City, CA). Detected mutations were confirmed by direct sequencing of re-amplified PCR products of bands with mobility shift as previously described [9].

RESULTS

Of 17 cases of BCC, 15 (88%) and 12 (65%) showed nuclear staining in tumor nests with CM1 and DO7, respectively. Of the 17 cases, 11 (65%) and seven (41%) showed nuclear staining in the epidermis adjacent to BCC with CM1 and DO7, respectively **(Table I)**. The stained cells were occasionally clustered in small areas of the epidermis **(Fig 1)**. All cases with nuclear staining in the epidermis also showed that in tumor nests. All cases showing reactivity with DO7 were stained with CM1 as well.

Because all BCC specimens examined here were obtained from facial lesions that were usually exposed to sunlight, the frequent p53 overexpression detected in the epidermis adjacent to BCC might be related to chronic UV light exposure. To test this possibility, p53 expression was examined in normal skin obtained from various sites. Of 16 specimens obtained from sites with usual sun exposure, 11 (69%) and seven (44%) showed nuclear staining in

Table II. Oligonucleotide Primers to Amplify Exons 2 to 10 of the p53 Gene

Exon	Sense Primer	Antisense Primer
2–3	5′-AGACTGCCTTCCGGGTCACT-3′	5′-AACCCTTGTCCTTACCAGAA-3′
4	5′-AGGACCTGGTCCTCTGACTG-3′	5′-GCATTGAAGTCTCATGGAAG-3′
5	5′-TTCCTCTTCCTACAGTACTC-3′	5′-CAGCTGCTCACCATCGCTAT-3′
6	5′-CACTGATTGCTCTTAGGTCT-3′	5′-AGTTGCAAACCAGACCTCAG-3′
7[a]	5′-GTGTTA(G)TCTCCTAGGTTGGC-3′	5′-CAAGTGGCTCCTGACCTGGA-3′
8	5′-CCTATCCTGAGTAGTGGTAA-3′	5′-TCCTGCTTGCTTACCTCGCT-3′
9	5′-TTGCCTCTTTCCTAGCACTG-3′	5′-CCCAAGACTTCGTACCTGAA-3′
10	5′-CTCTGTTGCTGCAGATCCGT-3′	5′-GCTGAGGTCACTCACCTGGA-3′

[a] Two kinds of sense primers were used to amplify exon 7 of the p53 gene because this region contained a common polymorphic site as shown in a parenthesis [34].

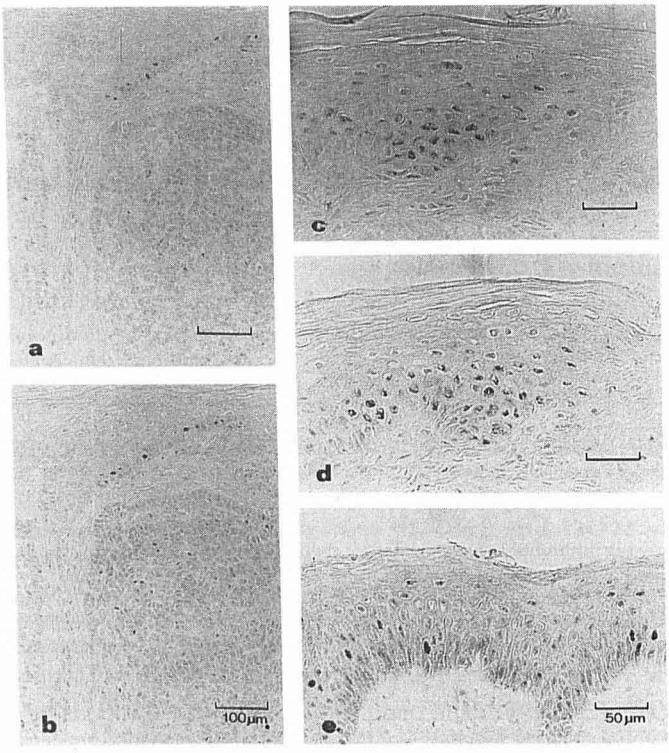

Figure 1. Immunohistochemical detection of p53 with CM1 (*a,c,e*) and DO7 (*b,d*). *a,b*) Some stained nuclei are present in the epidermis and tumor nests in a case of BCC. *c,d*) Stained nuclei are clustered in a small area in the epidermis adjacent to BCC in another patient. *e*) Stained nuclei are scattered in the normal epidermis with usual sun exposure. *Bar*, 100 μm (*a,b*) and 50 μm (*c,d,e*).

the epidermis with CM1 and DO7, respectively **(Fig 1 and Table I)**. Unlike those in the epidermis adjacent to BCC, stained cells were scattered in the normal epidermis instead of forming a cluster. Of 11 specimens obtained from sites with rare sun exposure, on the other hand, only 2 (18%) showed nuclear staining in a few cells of the epidermis with CM1, and none was stained with DO7 **(Table I)**.

In a subset of 13 different cases showing CM1 staining in the epidermis (10 cases of normal skin with usual sun exposure and three cases of BCC), mutations of the p53 gene were first screened by PCR-SSCP analysis. These 10 cases of normal skin were chosen randomly from those older than 50 years of age. Three cases of BCC were selected because epidermal portions stained with CM1 were separated enough (more than 2 mm) from tumor nests, so that DNAs of epidermal portions showing CM1 staining and those of tumor cells could be separately prepared without cross-contamination. The age of these three patients was, respectively 58, 63, and 76 years. The epidermis adjacent to BCC showed no histologic atypia in these three cases. No mobility shift in PCR-SSCP analysis was found in exons 2 to 10 of the p53 gene in 10 DNAs of the normal epidermis with usual sun exposure. All three DNAs of the epidermis adjacent to BCC showed mobility shifts in exon 7 **(Fig 2)**. DNA sequencing of bands showing altered migrations revealed a missense mutation of C to G transversion at a dipyrimidine site of codon 249 in all three DNAs, which replaced arginine with threonine **(Fig 3)**. Presence of this mutation was supported by loss of a *Hae*III site in DNAs showing mobility shifts (data not shown). An analysis of a microsatellite marker (D11S527) by PCR showed different patterns in each DNA carrying the mutation (data not shown). Therefore it was unlikely that the presence of the same mutation in these three samples was caused by cross-contamination. To rule out a possibility of contamination of PCR products, PCR-SSCP analysis of exon 7 was performed in the three cases using DNAs re-prepared from three tissue sections consecutive to

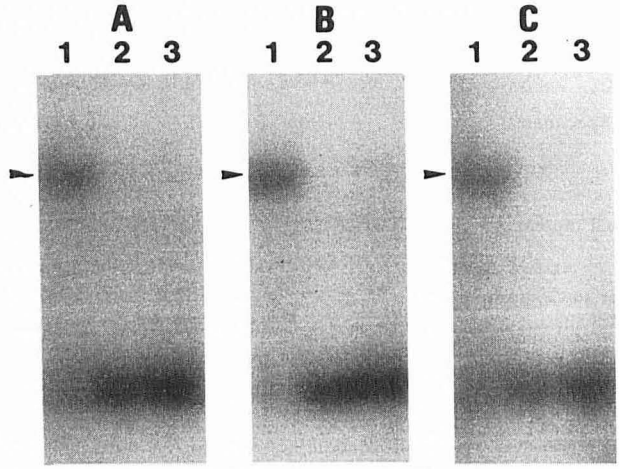

Figure 2. SSCP analysis of exon 7 of the p53 gene of three cases of BCC (*A, B, and C*). Electrophoresis was performed in an 8% polyacrylamide gel containing 10% glycerol. *Lane 1*, DNA from the epidermis adjacent to BCC; *lane 2*, BCC DNA; *lane 3*, peripheral blood cell DNA. *Arrowhead*, a band with a mobility shift.

those used for initial DNA preparation. An abnormal band showing a mobility shift was found in two of the three samples (data not shown), indicating that at least two samples carried p53 mutation. In one of these three cases of BCC, tumor cells had a missense mutation of C to T transition at codon 177 in exon 5, which substituted leucine for proline (data not shown). We have previously reported this case [9] and confirmed here the presence of the mutation in DNA obtained from a different portion of this BCC. No mutation was detected in exon 7 of these three DNAs from BCC.

DISCUSSION

In this study, immunohistochemical p53 overexpression was frequently detected in the normal epidermis with usual sun exposure compared to that with rare sun exposure ($p < 0.05$). Similar results have been reported recently [23]. Nuclear accumulation of p53 in *in vitro* cultured cells was induced after treatment with DNA-damaging agents including UV light irradiation [18]. Recent *in vivo* studies also demonstrated p53 overexpression in the epidermis after acute injury by UV light irradiation [16,17]. Based on these, we hypothesize that chronic injury by UV exposure in daily life is the main contributor to p53 accumulation in normal epidermis with usual sun exposure.

Although immunohistochemical p53 overexpression is often correlated with p53 gene mutations [13], no mutation in exons 2 to 10 of the p53 gene was detected here in the normal epidermis with chronic sun exposure by PCR-SSCP analysis. Mutations of the p53 gene in sun-exposed normal skin have been reported recently using very sensitive methods of mutant allele-specific PCR and mutant allele-specific ligase chain reaction [24]. These methods might detect mutations that are not clonally expanded and are present in cells incapable of growth. PCR-SSCP analysis used here is much less sensitive than these methods. Therefore, our results do not necessarily mean the absence of p53 gene mutations in normal epidermal cells with sun exposure, but do suggest rarity of clonal expansion of p53 mutant cells in the normal epidermis with sun exposure.

In the present study, accumulation of p53 was also frequently detected in the epidermis adjacent to BCC developing on the face that was usually sun-exposed. In the epidermis adjacent to BCC, unlike normal epidermis with usual sun exposure, a missense mutation of C to G transversion was found at codon 249 in exon 7 of the p53 gene in three cases examined. This mutation was not due to an allelic polymorphism, because it was not present in leukocyte DNAs of the patients. Detection of the mutation by PCR-SSCP

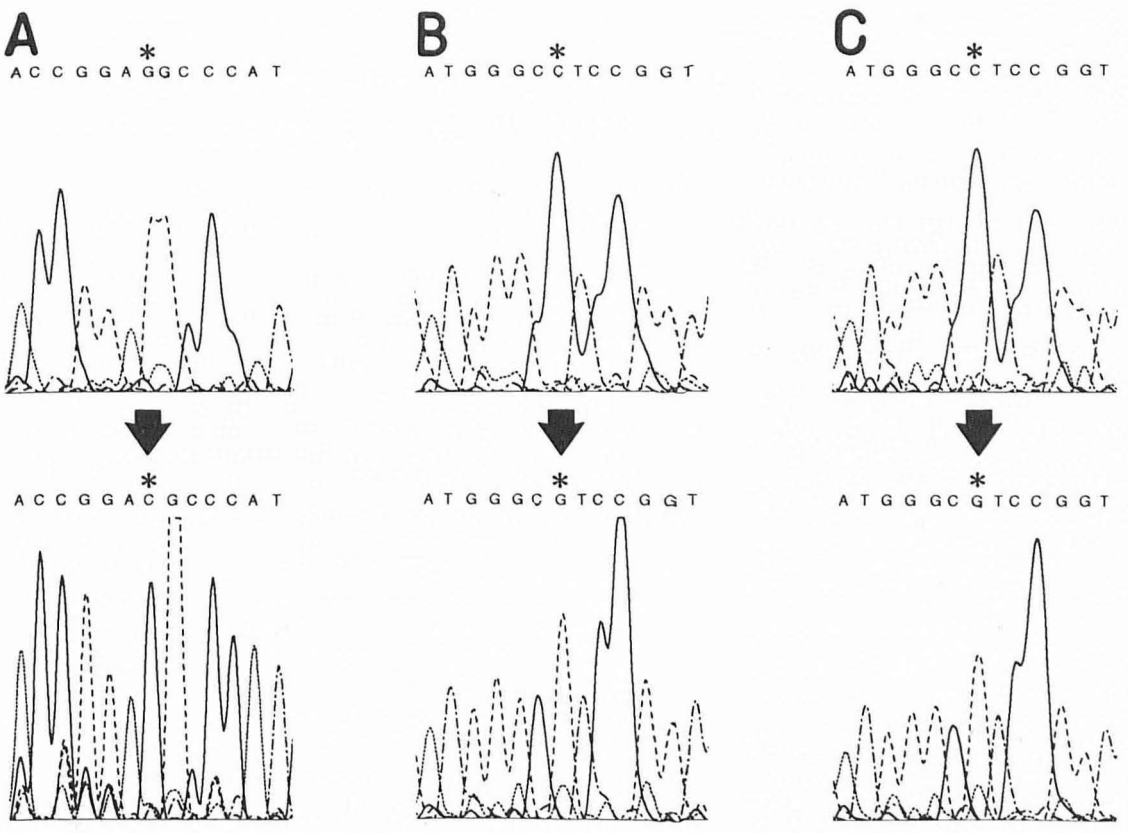

Figure 3. DNA sequences surrounding codon 249 in exon 7 of the p53 gene. *A*, *B*, and *C* correspond to those in **Fig 2**. Sequences of a sense strand and an antisense strand are shown in *A*, *B*, and *C*, respectively. *Top*, the normal sequence of peripheral leukocyte DNA; *bottom*, mutated sequences of DNAs from the epidermis adjacent to BCC. *Asterisk*, the base at which mutation was detected.

strongly suggested clonal expansion of p53 mutant cells in the epidermis adjacent to BCC of the three examined cases. This clonal expansion was limited to a small area, because the mutation was not detected in one case using DNA re-prepared from portions close to those used for initial DNA preparation. Presence of a small cluster of stained cells in the epidermis adjacent to BCC may provide immunohistochemical evidence of small clonal expansion of p53 mutant cells.

It is reasonable to speculate that the mutation may be relevant to skin carcinogenesis, firstly because codon 249 is located in an evolutionarily conserved domain of the p53 gene [4], secondly because this codon is a mutational hotspot in hepatocellular carcinomas [4], and lastly because a mutant p53 gene in this codon has been shown to induce mitotic activity in hepatoma cells [25]. Follow-up studies of patients with a history of BCC have indicated that they have high risk of developing subsequent new skin cancers [26]. Presence of clonally expanded p53 mutant cells in the epidermis of patients with BCC may provide a molecular basis of the high risk. Although mutations of codon 249 have been reported in three cases of skin cancers so far [6,9,27], the alterations do not appear to occur frequently in skin cancers. The discrepancy between frequent detection of the mutation at codon 249 in the epidermis adjacent to BCC and infrequent occurrence of the mutation in skin cancers remains to be answered.

It has previously been reported that the number of replicating cells are increased in the epidermis above BCC [28]. This observation is consistent with the presence of p53 mutations in the epidermis adjacent to BCC, because cells carrying certain p53 mutant genes obtain growth advantage by "gain of function" [25,29].

All mutations detected in the epidermis adjacent to BCC were a missense mutation of C to G transversion at a dipyrimidine site of codon 249 of the p53 gene. Although occurrence of mutations at a dipyrimidine site suggests involvement of UV light in mutagenesis [30], the two major types of DNA photoproducts, cyclobutane pyrimidine dimers and pyrimidine(6-4)pyrimidone photoproducts, which are potentially mutagenic [30], do not appear to be frequently formed at codon 249 of the p53 gene by UV irradiation [31]. However, this does not necessarily indicate lack of involvement of UV light in mutagenesis at this site, because a UV-specific mutation of CC to TT transition has been detected frequently at codons 245 and 247/248 in sun-exposed normal skin [24], where the photoproducts are infrequently formed [31]. Repair rates of pyrimidine dimers along the p53 gene have been recently demonstrated to be highly variable at individual nucleotides. A slow repair rate at these sites may contribute to frequent mutations [32]. Although pyrimidine dimers at codon 249 appear to be rapidly repaired in fibroblasts under contact inhibition [32], it will be the subject of future study to determine the repair rate at this site in the skin of patients with BCC, because repair capacities of UV-damaged DNAs have been demonstrated to be reduced in the skin of patients with BCC [33]. Repair rates of the (6-4) photoproducts also remain to be determined at nucleotide resolution along the p53 gene.

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