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Mutations of the *INK4a* Locus in Squamous Cell Carcinomas of Human Skin

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The *INK4a* locus encodes two different, cell cycle-regulating proteins, $p16^{INK4a}$ and $p19^{ARF}$. In this study, we screened mutations in all coding regions of the *INK4a* locus (exons 1β, 1α, 2, and 3) in 21 squamous cell carcinomas (SCCs) of human skin by polymerase chain reaction–single strand conformation polymorphism analysis. Mutations were detected in 3 SCCs in exon 2, which is common to both $p16^{INK4a}$ and $p19^{ARF}$. These included an in-frame deletion of 21 base pairs from codon 84 to 90, a frameshift mutation of CCC→TC at codon 75, and a nonsense mutation of CGA→TGA at codon 80 of the $p16^{INK4a}$ gene. These results suggest that inactivation of the *INK4a* locus has some relevance to the carcinogenesis in at least some of SCCs of human skin. This is the first demonstration of aberrations in the *INK4a* locus in SCCs of human skin. © 1997 Academic Press

Tumorigenesis is a multistep process that involves activation of protooncogenes and/or inactivation of tumor suppressor genes (1, 2). Mutations of the p53 tumor suppressor gene have frequently been observed in squamous cell carcinoma (SCC) of human skin (3-7). However it is unlikely that p53 gene mutations are sufficient for the development of SCC of human skin, because these gene mutations have been detected in apparently normal human epidermis (8-11). Additional genetic changes are required for the occurrence of SCC. Mutations of the *ras* genes have also been detected in SCC, but the frequency is low (12). A recent observation of frequent loss of heterozygosity (LOH) of a 9p21 chromosomal marker in human cutaneous SCCs may suggest the presence of tumor suppressor genes relevant to the development of SCC at the chromosomal locus (13).

$p16^{INK4a}$ is an inhibitory protein of cyclin-dependent kinase (CDK) 4, which regulates the cell cycle (14-16). The $p16^{INK4a}$ gene resides at chromosome 9p21 (15, 16). Germline mutations of this gene in familial melanoma and its homozygous deletions in a variety of human cancers, including sporadic melanoma, indicate that the $p16^{INK4a}$ gene is a tumor suppressor gene (15-20). Frequent LOH of a 9p21 marker in SCCs of human skin (13) may indicate the in volvement of the $p16^{INK4a}$ gene in skin carcinogenesis. However, the $p16^{INK4a}$ gene itself has not yet been studied in SCC of human skin.

The locus of the $p16^{INK4a}$ gene, named *INK4a*, has the capacity to produce two distinct transcripts from different promoters (21-23). Each transcript has a specific 5′ exon, exon 1α or 1β, which is spliced into common exons 2 and 3. Reading frames in exons 2 and 3 are different between the two transcripts. The exon 1α-containing transcript encodes $p16^{INK4a}$ (14-16). The exon 1β-containing transcript encodes $p19^{ARF}$, which also has the ability to arrest cell proliferation at both G1 and G2 of the cell cycle, albeit through mechanisms not involving direct inhibition of known CDK-cyclin complexes (23).

In this study, we examined 21 human cutaneous SCCs for mutations in all coding regions in the *INK4a* locus (exons 1β, 1α, 2, and 3) by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis. Mutations were confirmed by direct DNA sequencing of bands showing mobility shifts in PCR-SSCP analysis.

MATERIALS AND METHODS

Specimens. A total of 21 specimens of SCC were obtained from 21 patients. These included 14 well-differentiated, 4 moderately-differentiated, and 3 poorly-differentiated SCCs. Of these 21 SCCs, 19 were primary lesions and 2 were recurrent lesions. Fifteen SCCs originated in sunlight-exposed skin regions. The other 6 SCCs were assumed to be minimally related to UV light, including 3 SCCs that originated in scar tissue, 1 SCC that arose in radiation dermatitis, 1 SCC that occurred in the femoral region, and 1 SCC that occurred in the external genital region. All these specimens were previously used for p53 gene mutation analysis (7).

DNA preparation and PCR-SSCP analysis. DNAs of SCC were prepared from 19 frozen and 2 paraffin-embedded samples as pre-

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TABLE 1

Oligonucleotide Primers Used for PCR and DNA Sequencing

Exon	sense primer	Antisense primer	Annealing temp. (°C)
for PCR			
1β	GGTCCCAGTCTGTAGTTAAG	ACACCAAACAAAACAAGTGC	57
1α	GGGAGCAGCATGGAGCCG	AGTCGCCCGCCATCCCCT	62
2	AGCTTCCTTTCCGTCATGC	GCAGCACCACCAGCGTG	59
2	AGCCCAACTGCGCCGAC	CCAGGTCCACGGGCAGA	59
2	TGGACGTGCGCGATGC	GGAAGCTCTCAGGGTACAAATTC	57
3	CCGGTAGGGACGGCAAGA	CTGTAGGACCCTCGGTGACTGATGA	62
for DNA sequencing			
2	AGTGGCGGAGCTGCTGCT	GCAGCACCACCAGCGTG	57

Base sequences are represented from the 5′ to the 3′ ends. All primer sequences for PCR, except those for exon 1β, are the same as those described previously (17).

viously reported (7). Normal tissue DNAs were obtained from either the peripheral blood cells or the dermal portions of tissue sections surrounding the tumors in each patient. PCR was performed using the oligonucleotide primers listed in Table 1. SSCP analysis was performed with a 5% to 8% polyacrylamide gel containing 0% or 10% glycerol. Since the PCR products (344 base pairs) of exon 1β were too large for SSCP analysis, they were digested with a restriction enzyme, *Msp*I, before loading them on a gel. Analysis by PCR-SSCP was repeated at least twice to ensure that the results were reproducible in each case showing a mobility shift.

Direct DNA sequencing. Bands with mobility shifts in SSCP analysis were eluted from a gel and re-amplified by PCR with the same primers as used for the first PCR. After purification by means of spin filtration on a SUPREC-2 (Takara Shuzo, Kyoto, Japan), the re-amplified PCR products were subjected to direct DNA sequencing with a dsDNA Cycle Sequencing System kit (Life Technologies, Inc., Gaithersburg, MD, USA). Products were electrophoresed on a 6%, 8%, or 20% polyacrylamide gel containing 7 M of urea. Oligonucleotide primers used for DNA sequencing are shown in Table 1.

Analysis of loss of heterozygosity (LOH). Four microsatellite markers, *D9S162, D9S171, c5.1,* and *c1.B* (15), were used to detect LOH on chromosome 9p21. Analysis of LOH was performed as previously reported (24).

RESULTS

Mutations in exons of the *INK4a* locus, including exons 1β, 1α, 2, and 3, were screened by PCR-SSCP analysis. None of 21 SCC DNAs showed a mobility shift in exons 1β, 1α, and 3, although allelic polymorphism of exon 3 was detected in 1 DNA sample. On the other hand, 3 of 21 DNAs showed mobility shifts in exon 2 (Fig. 1). Apparent loss of normal bands was not detected in any of these 3 cases showing mobility shifts.

Direct DNA sequencing of bands showing mobility shifts in the three DNAs revealed an in-frame deletion of 21 base pairs from codon 84 to 90 in one of them, a frameshift mutation of CCC→TC at codon 75 in another, and a nonsense mutation of CGA→TGA at codon 80 of the $p16^{INK4a}$ gene in the third (Fig. 2). The deletion of 21 base pairs was supported by the presence of a shorter band in electrophoresis of PCR products on a 4% Nusieve agarose gel (data not shown). Table 2 sum-

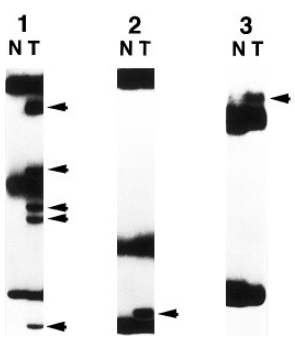

FIG. 1. SSCP analysis of exon 2 of the *INK4a* locus. Electrophoresis was performed in a 6% polyacrylamide gel containing 10% glycerol. The results obtained from three SCCs are represented, all of which have bands showing mobility shifts (arrowheads). Numbers at the top of the panel correspond to those in Table 2 and Fig. 2. All bands showing mobility shifts in panel 1 had an identical sequence after re-amplification by PCR. N, normal tissue DNA; T, SCC DNA.

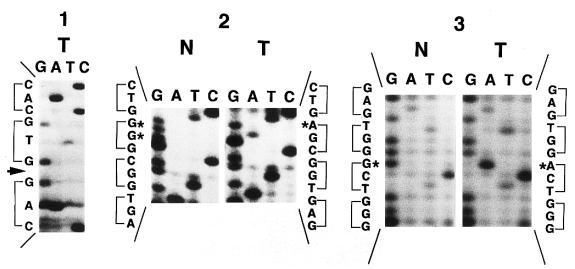

FIG. 2. DNA sequencing of exon 2 of the *INK4a* locus. Results using an antisense primer listed in Table 1 are shown. Therefore, sequences represented here are those of the transcribed strand. Twenty percent and 6% polyacrylamide gels containing 7 M urea were used in panel 1 and in panels 2 and 3, respectively. An arrowhead in panel 1 indicates a position of an in-frame deletion of 21 base pairs from codon 84 to 90 of the $p16^{INK4a}$ gene. In panel 2, a frameshift mutation of CC→T at codon 75 (GG→A in the transcribed strand) is shown. In panel 3, a nonsense mutation of C→T transition at codon 80 (G→A in the transcribed strand) is shown. Asterisk, the base at which mutation was detected.

TABLE 2

Summary of Mutations Detected in the *INK4a* Locus

Case	Age (sex)	Site	Exon codon[a]	Sequence[b]	Base change	Amino acid change	Predisposing conditions
1	63 (F)	Head	Exon 2 84-90		Deletion of 21 base pairs		Sun exposure
2	76 (M)	Cheek	Exon 2 75	acCCcg	CC → T	Frameshift	Sun exposure
3	48 (F)	Lower limb	Exon 2 80	ccCga	C → T	Arg → stop	Scar

[a] Condons are numbered as reported (19).
[b] Uppercase letters show nucleotide positions at which mutations were detected.

marizes the three mutation cases. The frameshift mutation and the 21-base pair deletion were found in poorly- and well-differentiated SCCs, respectively, both of which originated in sunlight-exposed skin regions. The nonsense mutation was detected in a well-differentiated SCC occurring in scar tissue on the lower leg.

Analysis of LOH using four microsatellite markers from 9p21 (*D9S162*, *D9S171*, *c5.1*, and *c1.B*) was not informative in the three cases with mutation because all these markers were homozygous in normal tissue DNAs (data not shown).

DISCUSSION

Frequent LOH of a microsatellite marker from chromosome 9p21 has recently been demonstrated in SCCs of human skin (13). This observation suggests that a tumor suppressor gene that is important in the development of SCCs resides at the chromosomal region. The *INK4a* locus is a potential candidate for the suppressor of SCC, because this locus maps to 9p21 (15, 16) and functions to suppress neoplastic growth (25). Although the most common mechanism of inactivation of the *INK4a* locus in human cancers is through homozygous deletion (26), intragenic mutations have been reported in primary tumors and tumor cell lines, including melanoma, SCC of the esophagus, and SCC of the head and neck (27-29). In this study, we detected intragenic mutations in the *INK4a* locus in 3 of 21 SCCs of human skin. To our knowledge, this is the first demonstration of alterations in the *INK4a* locus in SCCs of human skin. Although our analysis of LOH using four microsatellite markers from 9p21 failed to disclose a second hit on the *INK4a* locus in the 3 SCCs, we believe that inactivation of the locus was involved in the development of the SCCs.

Three mutations detected in this study comprised an in-frame deletion of 21 base pairs from codon 84 to 90, a frameshift mutation of CCC→TC at codon 75, and a nonsense mutation of CGA→TGA at codon 80 of the $p16^{INK4a}$ gene. Mutations in the *INK4a* locus that have been identified in human cancers to date affect $p16^{INK4a}$ either exclusively or together with $p19^{ARF}$ (23). All 3 mutations detected here affect both $p16^{INK4a}$ and $p19^{ARF}$. The aberration more relevant to the skin carcinogenesis, $p16^{INK4a}$ or $p19^{ARF}$ or both, remains to be determined.

The compilation of $p16^{INK4a}$ gene mutations identified in human cancers has shown that inactivation of the $p16^{INK4a}$ gene is frequently due to truncation of the protein through a combination of frameshifts, nonsense mutations, and splicing anomalies (26). Of the three mutations detected here, one was predicted to change the reading frame of the $p16^{INK4a}$ gene and lead to the premature introduction of a stop codon. Another mutation was assumed to produce a truncated $p16^{INK4a}$ protein through a nonsense mutation. The remaining one showed an intragenic deletion. Thus missense mutations were not detected in SCCs of human skin. Taking frequent homologous deletion of the $p16^{INK4a}$ gene in other human cancers into account (26), many missense mutations may be rather insufficient for the inactivation of $p16^{INK4a}$ protein.

Tumor suppressor genes may offer a clue to identifying carcinogens of human cancers. Mutation analyses of the p53 tumor suppressor gene have shown predominant C→T transitions at dipyrimidine sites in skin cancers originating in the sunlight-exposed skin regions, indicating the involvement of ultraviolet (UV) light in mutagenesis (3, 6, 7). Of two *INK4a* mutations identified here in SCCs originating in the sunlight-exposed skin regions, one was a CC→T mutation (a combination of deletion and transition) at a dipyrimidine site and the other was a deletion of 21 base pairs. In contrast to p53 gene mutations, both types of mutations are not specific and uncommon to UV mutagenesis. Detection of these uncommon mutations in SCCs originating in the sunlight-exposed skin regions may be related to a biological property of $p16^{INK4a}$: this protein is not easily inactivated by missense mutations. This property constrains the ability of a base substitution to lead to a tumor, so that many $p16^{INK4a}$ mutations made by the original

carcinogen will not be found in tumors. Therefore, mutation analysis in the *INK4a* locus may not be helpful in identifying carcinogens of human cancers.

A third mutation detected here was a C→T transition at codon 80 of the $p16^{INK4a}$ gene, occurring at a dipyrimidine site (cCg→cTg). This was found in an SCC originating in scar tissue on the lower leg in a location that had rarely been exposed to sunlight. This mutation site was a CpG dinucleotide site, where mutations might be related to deamination of 5-methylcytosine (30). The same mutation has been identified in internal malignancies, suggesting that this site is one of the mutational hotspots within the *INK4a* locus (26).

We previously studied mutations of the p53 gene in the same 21 SCCs examined in this study (7). The previous study indicated that, of the three SCCs with mutations in the *INK4a* locus, one SCC arising in the scar tissue on the lower leg had a mutation of the p53 gene as well, and the two others had no p53 gene mutations. Coexistent mutations in both gene loci suggest that $p16^{INK4a}$ (and $p19^{ARF}$) and p53 proteins function in separate pathways, each of which is important in suppressing skin carcinogenesis. Inactivation of both $p16^{INK4a}$ and p53 proteins has also been detected in some other primary tumors (18, 29, 31).

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