BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **230,** 85–88 (1997)
ARTICLE NO. RC965891

# Loss of Expression of the p16 Gene Is Frequent in Malignant Skin Tumors

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Received November 26, 1996

**Expression of the p16 gene from 30 malignant skin tumors has been surveyed by immunohistochemical assay. Gene point mutations were detected by DNA direct sequencing and the mRNA level of gene expression was measured by RT-PCR. A silent point mutation of the p16 gene was found in only one patient. However, loss of expression of the p16 gene was noticed in 23 of 29 samples (79.3%). Correlation between loss of expression of the p16 gene and metastasis is significant ($p = 0.0036$). These findings suggest that loss of expression of the p16 gene may play a critical role in tumor progression of malignant skin tumors.** 

Cellular proliferation is promoted from G1 to S phase by cyclin D-cdk complexes (1). Activated cyclin D-cdk complexes can phosphorylate Rb protein and mediate the cell cycle progression (2). The p16 protein, encoded by the p16 (CDKN2, INK4A, or MTS1) gene, is an inhibitor of Cdk4. By binding to Cdk4 and inhibiting phosphorylation of the Rb protein, p16 could arrest cell cycle in G1 phase and suppress cell proliferation (3,4,5). Functional or structural loss of p16 could, therefore, lead the premalignant or malignant cell to undergo abnormal division. The p16 gene has been found to be mutated in some familial melanoma (6) and also in high frequency for non-small cell lung carcinomas (7), esophageal carcinoma (8), mesothelioma (9), leukemia (10), and pancreatic adenocarcinoma (11).

In this study, we have evaluated by immunohistochemistry the expression of p16 protein in 30 primary skin tumor samples. In 13 of those samples, we looked for point mutations of the p16 gene by direct sequencing PCR products of exon1 and exon2 of the gene. To measure the mRNA level of the p16 gene, we also performed RT-PCR analysis.

## MATERIALS AND METHODS

*Tumor specimens.* Thirty skin tumor specimens resected at Veterans General Hospital-Kaohsiung were used in the immunohistochemical study. Histological subtypes were as follows: acral lentiginous melanoma, 16; squamous cell carcinoma, 6; schwannoma, 4; angiosarcoma, 2; basal cell carcinoma, 1; adenocarcinoma, 1 (see Table 1). Fourteen of those cases were also studied by PCR, RT-PCR and direct sequencing of exons 1 and 2 of the p16 gene, which correspond to 97% of the entire coding region.

*Immunohistochemical staining.* Sections (2-4 $\mu$m thick) were cut from paraffin-embedded specimens, deparaffinized in xylene and rehydrated through a graded series of ethanol. The sections were treated with microwave to unmask the antigen and incubated at room temperature with the anti-p16 antibody, C-20 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Immunolocalization was performed with the LSAB kit(Dako) and amino-ethyl carbazole. Tissue sections were counterstained with hematoxylin and mounted in Glycerol (Dako) before observation under a standard microscope.

*PCR amplification and direct sequencing.* Genomic DNA was extracted from dissected frozen specimens by a standard protocol (12). Primers for exon 2 of the p16 gene (13) were used with those for the 9p marker. PCR reactions were performed under standard conditions, except for the presence of 5% dimethyl sulfoxide in exon 2 amplification. Annealing temperatures used were 58 °C for exon 1 and 55 °C for exon 2. Direct sequencing in both directions was accomplished using a *fmol* DNA Sequencing System (Promega).

*RT-PCR analysis p16 expression.* Gene expression was determined by PCR amplification of the full-length p16 gene using primers described by Jen et al (14). The integrity of RNA samples was evaluated by control PCR amplifications of human GAPDH using primer set for RT-PCR (Stratagene). Both primer sets yield products which span intron-exon junctions, ensuring that the products are mRNA-derived.

*Statistical analysis.* Fisher's exact test was used to analyze the correlation between gene expression of p16 and metastasis (15).

## RESULTS AND DISCUSSION

Thirty human malignant skin tumors were examined for expression of the p16 gene by immunohistochemical detection. In built-in normal control the p16 immuno-



0006-291X/97 $25.00

**TABLE 1**

Histologic Types and p16 of Malignant Skin Tumors

| | | p16 | | |
|---|---|---|---|---|
| Case[a] | Histologic type[b] | Gene (Direct sequencing)[c] | mRNA (RT-PCR)[d] | Protein (Immunohistochemistry)[e] |
| 1 | SCW | WT | ± | − |
| 2 | AS | a silent mutation | ± | ND |
| 3* | SCC | WT | − | − |
| 4 | SCC | WT | ND | − |
| 5 | ALM | WT | + | − |
| 6* | SCC | WT | ± | − |
| 7 | SCC | WT | + | + |
| 8 | SCC | WT | ± | − |
| 9 | ALM | WT | ± | − |
| 10 | SCC | WT | ± | + |
| 11 | BCC | WT | ± | − |
| 12 | ALM | WT | ND | + |
| 13* | ALM | ND | ± | − |
| 14* | AC | WT | ± | − |
| 15* | ALM | NA | NA | − |
| 16* | ALM | NA | NA | − |
| 17* | ALM | NA | NA | − |
| 18* | ALM | NA | NA | − |
| 19 | ALM | NA | NA | + |
| 20* | ALM | NA | NA | − |
| 21* | ALM | NA | NA | − |
| 22* | ALM | NA | NA | − |
| 23* | ALM | NA | NA | − |
| 24* | ALM | NA | NA | − |
| 25* | ALM | NA | NA | − |
| 26* | ALM | NA | NA | − |
| 27* | ALM | NA | NA | − |
| 28 | AS | NA | NA | − |
| 29 | SCW | NA | NA | + |
| 30 | SCW | NA | NA | + |

[a] *, metastasis.
[b] AC, adenocarcinoma; AS, angiosarcoma; ALM, acra lentiginous melanoma; BCC, basal cell carcinoma; SCC, squamous cell carcinoma; SCW, schwannoma.
[c] WT, wild-type; ND, not done; NA, not applicable.
[d] −, not detected; ± detectable at a minimal level; +, detectable.
[e] −, loss of expression; +, expression of p16 in nucleus.

histochemical assay showed moderate to strong nuclear staining and variable levels of cytoplasmic reactivity (Fig. 1A). Failure of nuclear p16 staining to appear can be taken as loss of p16 expression (16,17). Loss of expression of the p16 gene was detected in 23 of 29 malignant skin tumors (see Table 1); an example of such is shown in Fig. 1B. All skin tumors with metastasis, including 13 acral lentiginous melanoma, 2 squamous cell carcinoma, and 1 adenocarcinoma, were shown to have loss of expression of the p16 gene (see Table 1).

The exons 1 and 2 of the p16 gene were amplified from thirteen frozen malignant skin tumors, including cases with negative immunohistochemical staining. Both strands of exons were sequenced at least two times from different preparations of PCR products. It was found that only one point mutation, a silent mutation, occurred at the 167 nucleotide of exon 2 in case2.

RT-PCR was used to determine the expression of full-length p16 gene in normal and tumor tissues. The expressed product of p16 gene was observed in normal tissues and in case 5 tumor tissue (Fig. 2). All other tumor samples showed no products or barely detectable expressed products of p16 gene. This is in concordance with the results from the immunohistochemical study.

Recent experiments (18,19,20) have shown that abnormal DNA hypermethylation within exon1 of the p16 gene may result in transcriptional silencing in tumors that do not carry detectable structural p16 mutations. The loss of expression in messenger RNA and protein synthesis in most tumors leads us to suggest the possibility of this down-regulation mechanism.

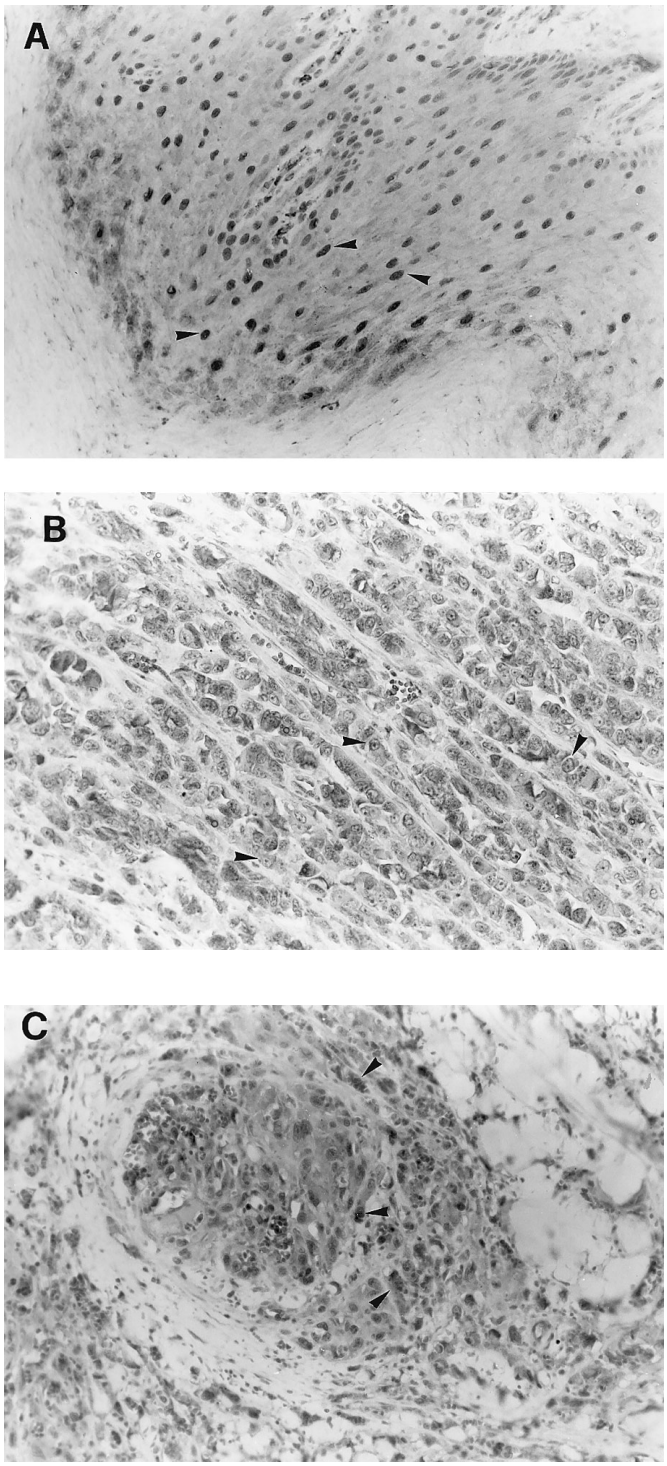


**FIG. 1.** Immunohistochemical detection of malignant skin tumors for p16 protein. (A) Immunolabeling of built-in normal epidermal cells, found to express p16 as evidenced by dark nuclear staining (arrowheads); ×150. (B) Metastatic melanoma showing complete loss of p16 expression as evidenced by lack of nuclear staining (arrowheads); ×150. (C) Expression of p16 in non-metastatic melanoma (case 19) showing nuclear staining in most of the tumor cells (arrowheads); ×150.

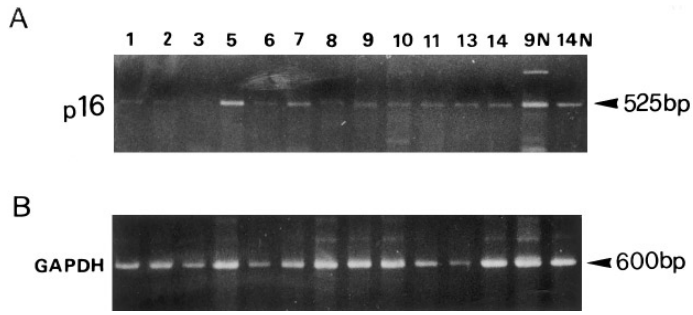


**FIG. 2.** Expression of the p16 gene. RT-PCR analysis was used to examine the expression of the p16 gene in dissected skin tumors. The case numbers are marked on the top. (A) RNA samples were incubated with reverse transcriptase and the cDNA products were used to PCR amplify the p16 gene. Ethidium bromide-staining agarose gel shows detectable p16 full-length gene product (p16) in case 5, case 7, and normal tissue counterparts of case 9 (9N) and case 14 (14N). (B) A standard "housekeeping" gene GAPDH is expressed in all samples tested.

In agreement with previous descriptions (17,21,22), we found that the correlation between the loss of expression of the p16 gene and the metastatic stage is significant ($p = 0.0036$). In summary, our results indicate that loss of expression of the p16 may play a critical role in tumor progression of human malignant skin tumors.

## ACKNOWLEDGMENTS

This work was supported by the VGH Research Program (VGHKS85-73 and VGHKS85-62) from Medical Research Advancement Foundation in Memory of Dr. Chi-shuen Tsou. We thank Professor Larry Steinrauf for reading the manuscript and helpful suggestions.

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