Immunohistochemical Detection of p16, p21, and TGF-β in Cutaneous Epithelial Tumors

Kee Woan Park, M.D., Jong Yuk Yi, M.D., Chul Jong Park, M.D., Si-Yong Kim, M.D.

Department of Dermatology, College of Medicine, The Catholic University of Korea, Seoul, Korea

Background : It has been known that p16, p21, and TGF-β are related to cellular proliferation and malignant transformation but the results of the previous studies are controversial.

Objective : This study was performed in order to investigate the possible role of p16, p21, and TGF-β in relation to the cellular proliferation and malignant transformation in various skin tumors.

Methods : For immunohistochemical staining we examined sections (4㎛ thick) of formalin-fixed, paraffin-embedded tissue from 11 cases of squamous cell carcinoma (SCC), 13 cases of basal cell carcinoma (BCC), 7 cases of actinic keratosis, 5 cases of keratoacanthoma, and 4 cases of normal skin for control. Following conventional deparaffinization, the three step immunoperoxidase method was performed using the streptavidine-biotin complex and monoclonal antibodies. All sections were counterstained with hematoxylin. The expression of p16, p21, and TGF-β was evaluated and graded in relation to the intensity of cytoplasmic immunostaining of positive epidermal cells.

Results : p16, p21, and TGF-β were detected mainly in the cytoplasm. The p21 positive cells were localized exclusively in the upper squamous layer and were not visualized in the basal layer. The expression of p21 was higher in SCC than BCC (P value=0.03). The expression of p21 in normal control was higher than skin tumors, but there was no significant difference among these tumors. There was a significant loss in the expression of p16 and TGF-β in various skin tumors compared with normal control, but no difference in the expression of p16 and TGF-β among these skin tumors.

Conclusion : 1. p21 is possibly related to the differentiation of epidermal cells, because p21 was observed not in basal cells but in squamous and granular cells of the normal epidermis and the higher expression of p21 was demonstrated in SCC compared to BCC.

2. It may imply that loss of expression of p16 may play a critical role in malignant transformation but not in tumor progression of human malignant skin tumors, because the significant loss of the expression of p16 was found in SCC and BCC when normal skin and there was no difference in the expression of p16 among various skin tumors but the expression of p16 of SCC is higher than that of actinic keratosis (P value=0.04).

3. It may imply that the loss of the expression of TGF-β may play a critical role in malignant transformation, because the significant loss of expression of TGF-β was found in various skin tumors when compared to normal skin, but there was no difference in the expression of TGF-β among various skin tumors. (Ann Dermatol 13(1) 22~27, 2001).

Key Words : p16, p21, TGF-β

Received March 28, 2000.
Accepted for publication October 24, 2000.
Reprint request to : Kee Woan Park, M.D.,
Department of Dermatology
College of Medicine, The Catholic University of Korea, Seoul, Korea

Cellular proliferation is promoted from G1 to S phase by cyclin D/CDK (cyclin dependent kinase) complex. p16 and p21 belong to the protein family of CDK inhibitors, which are important negative regulators of the cell cycle[1]. p16 is encoded by the

CDKN2A gene located on chromosome 9p21. p16 could arrest cell cycle in G1 phase and suppress cell proliferation due to the catalytic activity of the CDK4/cyclin D complex[2], and functional or structural loss of p16 could lead the premalignant and malignant cells to undergo abnormal division[3].

p21 is encoded by a p53-inducible gene on chromosome 6p21. p21 inhibits the function of G1 cyclin dependent kinases to phosphorylate the retinoblastoma protein and prevents entry of the cells into the S phase of the cell cycle by DNA damage[4,5]. In melanocytic tumors, p21 levels were found to be low or undetectable in majority of benign lesions, with greater p21 expression seen in malignant tumors[6].

TGF-β is a protein which appears to play a complex role in the control of cell growth and differentiation[7,8]. The growth of epithelial cells such as human keratinocytes, carcinoma, and melanoma cell lines is generally inhibited by TGF-β[8-10].

We investigated the expression of p16, p21 and TGF-β in relation to cellular proliferation and malignant transformation in various skin tumors.

MATERIALS AND METHODS

A total of 40 skin biopsy specimens are studied. The histologic diagnosis was as follows: 11 cases of squamous cell carcinoma, 13 cases of basal cell carcinoma, 7 cases of actinic keratosis, 5 cases of keratoacanthoma, and 4 cases of normal skin for control. For immunohistochemical stain, sections (4μm thick) were cut from paraffin-embedded specimens. After deparaffinized in xylene and rehydrated through a graded series of ethanol, the sections were placed in a jar filled with 10mmol/l citrate buffer, pH6, heated twice in a microwave oven for 5min and kept in citrate buffer for 20min at room temperature. They were then treated with 1% hydrogen peroxide solution containing methanol to inhibit endogenous peroxidase. The antibodies of p16, p21 (Pharmingen, San-Diego, CA, USA) and TGF-β (Serotec Ltd, Oxford, UK) were used at a dilution of 1:100 with overnight incubation at 4 oC and detection was performed by streptavidine-biotin peroxidase system (LSAB kit, Dako, Kyoto, Japan). 3-amino-9-ethyl carbazole (K696, Dako) was used as the chromogen. All sections were counterstained with hematoxylin. The expression of p16, p21, and TGF-β was evaluated and graded in relation to the intensity of cytoplasmic immunostaining and the proportion of positive cells. We classified immunoreactivity into six scales : absent (0), focal and weak staining (1), focal and moderate staining (2), diffuse and moderate staining (3), focal and intense staining (4), diffuse and intense staining (5). The specimens were assessed by two independent observers and there was a good correlation between them. Mann-Whitney exact test was used to analyze the expression of p16, p21,and TGF-β in various skin tumors.

RESULTS

p16, p21, and TGF-β were all detected mainly in the cytoplasm of normal, benign and malignant neoplastic cells. In the normal skin, the expression of p16, p21, and TGF-β were all positive and the p21 positive cells were localized exclusively in the upper squamous layer and were not visualized in the basal layer. The expression of p21 is higher in SCC than BCC (P value=0.03). The expression of p21 in normal control was higher than skin tumors but there was no significant difference among these tumors.

There was a significant loss in the expression of p16 observed in SCC (P value=0.02), actinic keratosis (P value=0.01), BCC (P value=0.01), and keratoacanthoma (P value=0.05) compared with normal skin. There was no difference in the expression of p16 among various skin tumors but the expression of p16 of SCC is higher than that of actinic keratosis (P value=0.04).

There was a significant loss in the expression of TGF-β observed in SCC (P value=0.05), actinic keratosis (P value=0.02), BCC (P value=0.03), and keratoacanthoma (P value=0.07) compared with normal skin. There was no difference in the expression of TGF-β among various skin tumors.

DISCUSSION

The cell cycle and the genetic alteration that drive tumorigenesis are linked. Examples include the amplification of cyclin and CDK genes, the control of CDK inhibitor p21 by p53, and the tumor suppressor activity of the CDK inhibitor p16. It has been known that p16, p21, and TGF-β are related to cellular proliferation and malignant trans-

formation but the results of the previous studies are controversial. Cellular proliferation is promoted from G1 to S phase by cyclin D/cyclin dependent kinase (CDK) complex. p16 and p21 belong to the protein family of CDK inhibitors, which are important negative regulators of the cell cycle[1]. p16 could arrest cell cycle in G1 phase and suppress cell proliferation due to the catalytic activity of the CDK4/cyclin D complex[2]. Functional or structural loss of p16 could, therefore, lead the premalignant and malignant cells to undergo abnormal division[3]. Differential expression of p16 protein has been reported in cutaneous melanocytic lesions with the loss of expression associated with the invasive stage of tumor development[6]. It was reported that loss of expression of p16 in malignant skin tumors was frequent whereas expression of p21 was lower in benign lesions[3,6]. And it has been suggested that loss of expression of p16 may play a critical role in tumor progression of human malignant skin tumors but not in malignant transformation[6]. In this study significant loss of expression of p16 was found in SCC and BCC compared with normal skin and the expression of p16 of keratoacanthoma and actinic keratosis was lower than normal skin. And there was no difference in the expression of p16 among skin tumors but the expression of p16 of SCC is higher than that of actinic keratosis. It may imply that loss of expression of p16 may play a critical role in malignant transformation but not in tumor progression of human malignant skin tumors.

p21, also known as WAF1, CIP1, SDI1, MDA6, or CAP20, is encoded by a gene on chromosome 6p and can be directly regulated by p53[11]. p21 binds to multiple cyclin/CDK complexes and proliferating cell nuclear antigen to control progression through different phases of the cell cycle[3,4]. There is a possibility of p21 being concerned with the pro-

Table 1. Immunoreactivity of p16

		Immunoreactivity					
	Case	0	1	2	3	4	5
Normal	4			1		2	1
AK	7	4	1	1	1		
KA	5	1		4			
SCC	11		2	7	2		
BCC	13		5	7	1		

Table 2. Immunoreactivity of p21

		Immunoreactivity					
	Case	0	1	2	3	4	5
Normal	3			1			2
AK	7		2	4	1		
KA	5			4		1	
SCC	11		3	5		3	
BCC	13	2	7	2	1		

Table 3. Immunoreactivity of TGF-β

		Immunoreactivity					
	Case	0	1	2	3	4	5
Normal	3			1	1	1	
AK	7	2	4	1			
KA	5	1	3		1		
SCC	11	6	3		1	1	
BCC	13	7	2	2	2		

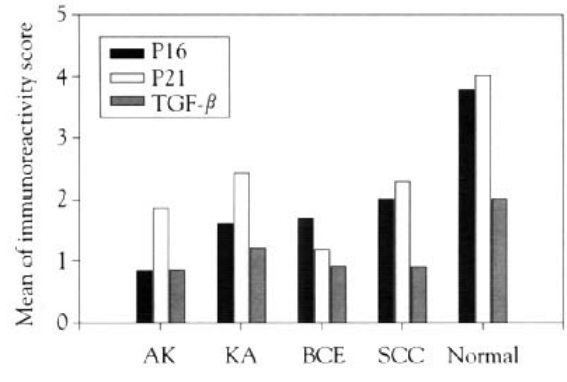

Figure 1. Comparison of immunoreactivity of p16, p21, TGF-βamong various skin tumors. The expression of p21 is higher in SCC than BCC (P value=0.03). The expression of p21 in normal control was higher than skin tumors but there was no significant difference among them. There was a significant loss in the expression of p16 observed in SCC (P value=0.02), actinic keratosis (P value=0.01), BCC (P value=0.01), and keratoacanthoma (P value=0.05) compared with normal skin. There was a significant loss in the expression of TGF-βobserved in SCC (P value=0.05), actinic keratosis (P value=0.02), BCC (P value=0.03), and keratoacanthoma (P value=0.07) compared with normal skin. There was no difference in the expression of p16 and TGF-βamong various skin tumors.

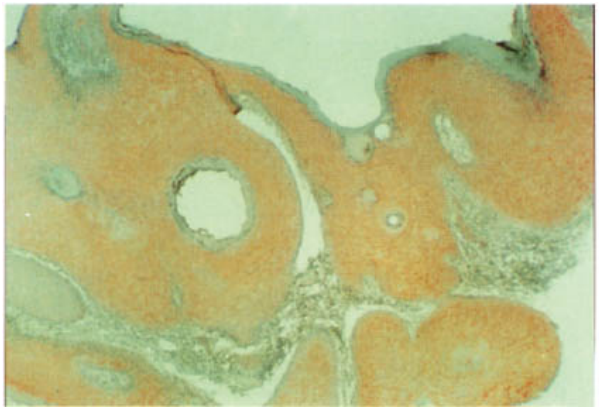

Fig 2. p21 expression in keratoacanthoma. Diffuse and moderate cytoplasmic positivity (scale 3) is seen in almost tumor cells.

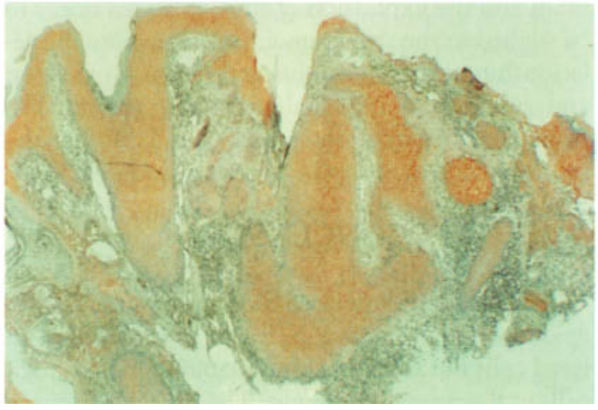

Fig 3. p16 expression in squamous cell carcinoma. Diffuse and moderate cytoplasmic positivity (scale 3) is seen in almost tumor cells.

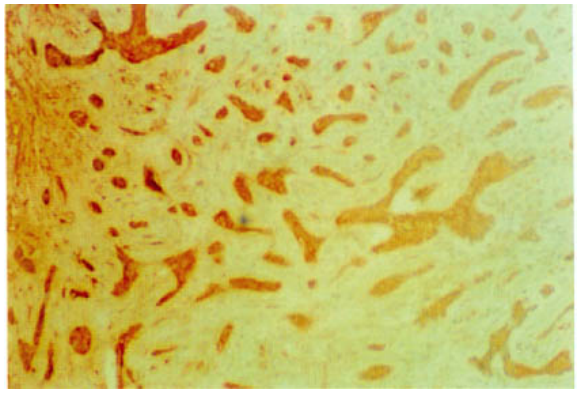

Fig 4. TGF-βexpression in basal cell carcinoma. Diffuse and moderate cytoplasmic positivity (scale 5) is seen in almost of tumor cells.

liferation and differentiation of cancer cells. According to many recent immunohistologic studies, p21 was found in cancer cells but within those tissues expression was often sharply restricted to cells at specific stages of differentiation : terminally differentiated cells generally showed stronger reactivity with antibody to p21[12-13]. In melanocytic tumors, p21 levels were found to be low or undetectable in the majority of benign lesions with greater p21 expression seen in malignant tumors[6]. In the present experiment, p21 was not observed in basal cells but was found in squamous and granular cells in the normal epidermis. This suggests that p21 is possibly related to the differentiation of epidermal cell. And comparing the findings of immunoreaction between BCC and SCC groups, the higher expression was demonstrated in the latter probably due to the high percentage of the well differentiated component. There was no difference in the expression of p21 among SCC, actinic keratosis, and keratoacanthoma, and this may indicate that p21 expression is not related to cell proliferation and malignant transformation.

TGF-β is a protein which appears to play a complex role in the control of cell growth and differentiation[7,8]. The growth of epithelial cells such as human keratinocytes, carcinoma, and melanoma cell lines is generally inhibited by TGF-β[8-10] and the suppression of TGF-β activity seems to be a likely mechanism of epithelial carcinogenesis[14,15]. Paradoxically expression of TGF-β appears to increase in several types of tumors and premalignant lesions[16,17]. In this study, significant loss of expression of TGF-β was found in all the skin tumors compared with normal skin but there was no difference in the expression of TGF-β among skin tumors. It may also imply that loss of expression of TGF-β may play a critical role in malignant transformation.

Taken together, the results of this study that p21 is possibly related to the differentiation of epidermal cells and loss of TGF-β may play a critical role in malignant transformation are in agreement with previous description[12-13,16-17]. The result of this study that loss of p16 may play a critical role in malignant transformation, not in tumor progression of malignant skin tumors is contrary to other previous studies[3,6]. It is unclear why the result of p16 is opposite to other studies. The sample size of this study was

small and the expression of these proteins needs to be studied in the same tumor to demonstrate the relationship of these proteins and tumor progression and differentiation of malignant skin tumors. We think that the role of p16, p21 and TGF-β of the skin tumors remains to be further elucidated.

CONCLUSION

1. p21 is possibly related to the differentiation of epidermal cell because p21 was not observed in basal cells but was found in squamous and granular cells in the normal epidermis. The higher expression of p21 was demonstrated in SCC compared to BCC probably due to the high percentage of the well differentiated component. There was no difference in the expression of p21 among SCC, actinic keratosis, keratoacanthoma and this may indicate that p21 expression is not related to cell proliferation and malignant transformation.

2. Significant loss of expression of p16 was found in SCC and BCC compared with normal skin. And there was no difference in the expression of p16 among various skin tumors but the expression of p16 of SCC is higher than that of actinic keratosis (P value=0.04). It may imply that the loss of expression of p16 may play a critical role in malignant transformation but not in tumor progression of human malignant skin tumors.

3. Significant loss of expression of TGF-β was found in various skin tumors compared with normal skin but there was no difference in the expression of TGF-β among various skin tumors. It may imply that the loss of expression of TGF-β may play a critical role in malignant transformation.

REFERENCE

1. Sherr CJ, Roberts JM: Inhibitors of mammalian G1 cyclin-dependent kinases. Genes Dev 9:1149-1163, 1995.
2. Serrano M, Hannon GJ, Beach D: A new regulatory motif in cell cycle control causing specific inhibition of cyclin D/CDK4. Nature 366:704-707, 1993.
3. Chang TG, Wang JS, Chen LW, et al: Loss of expression of the p16 gene is frequent in malignant skin tumors. Biochem. Biophys. Res. Comm 230:85-88, 1977.
4. Xiong Y, hannon GJ, Zhang H, Casso D, Kobayashi R, Beach D: p21 is a universal inhibitor of cyclin kinases. Nature 366:701-704, 1993.
5. Murray AW: creative blocks: cell-cycle checkpoints and feedback controls. Nature 359:599-604, 1992.
6. Sparrow LE, Eldon MJ, English DR, Heenan PJ: p16 and $p21^{WAFI}$ protein expression in melanocytic tumors by immunohistochemistry. Am J Dermatopathol 20:255-261, 1998.
7. Assoian RK, Komoriya A, Meyers CA, Miller DM, Sporn MB: Transforming growth factor-β in human platelets. Identification of a major storage site, purification and characterization. J Biol Chem 258:7155-7160, 1983.
8. Moses HL, Tucker RF, Leof EB, Coffey RJ, Halper J, Shipley GD: Type-β transforming growth factor is a growth stimulator and a growth inhibitor. In Feramisco j, Ozanne B, Stiles C (eds): Cancer cells, Vol. 3, Cold Spring Harbor , NY: Cold Spring Harbor Laboratory, 1985.
9. Shipley GD, Pittelkow MR, Willie JJ, Scott RE, Moses HL: Reversible inhibition of normal human prokeratinocyte proliferation by typeβ transforming growth factor-growth inhibitor in serum free medium. Cancer Res 46:2068-2071, 1986.
10. Coffey RJ, Sipes Nj, Bascom CC, Graves-Deal R: growth modulation of mouse keratinocytes by trasforming growth factors. Cancer Res 48:21596-1602, 1988.
11. El-Deiry WS, Tokino T, Velculescu VE, et al: WAF1, a potential mediator of p53 tumor suppression. Cell 75:817-825, 1993.
12. Steinman RA, Hoffman B, Iro A, et al: Induction of p21 (WAF1/CIP1) during differentiation. Oncogene 9:3389-3396, 1994.
13. Jiang H, Lin J, Su ZZ, Collart Fr, Huberman E, Fisher PB: induction of differentiation in human promyelocytic HL-60 leukemia cells activates p21, WAF1/CIP1 expression in the absence of p53. Oncogene 9:3397-3406, 1994.
14. Takahashi K, Suzuki K, Ono T: Loss growth inhibitory activity of TGF-beta toward normal human mammary epithelial cells grown within collagen gel matrix. Biochem Biophys Res Commun 173:1239-1247, 1990.
15. Wyllie FS, Dawson T, Bond JA et al: Correlated abnormalities of transforming growth factor beta 1 response and p53 expression in thyroid epithelial cell transformation. Moll Cell Endocrinol 76:13-21, 1991.
16. Arrick BA, Lopez AR, Elfman F, Ebner R, Damsky

CH, Derynck R: Altered metabolic and adhesive properties and increased tumorigenesis associated with increased expression of transforming growth factor beta 1. J Cell Biol 118:715-726, 1992.

17. Saxena A, Ali IU: Increased expression of genes from growth factor signaling pathways in glioblastoma cell lines. Oncogene 7:243-247, 1992.