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Expression of p63, bcl-2, bcl-6 and p16 in Basal Cell Carcinoma and Squamous Cell Carcinoma of the Skin

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Background : Basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) are the common malignant neoplasms of the skin. The *p63* is a *p53* homologue which is considered to be a reliable keratinocyte stem cell marker. *Bcl-2* plays a key role in cell longevity by preventing apoptosis, whereas the *bcl-6* gene functions as a transcriptional repressor. The p16-CDK4/6 complex arrests the cell cycle at G_0/G_1 phase. In the present study, the expression of p63, bcl-2, bcl-6, and p16 in BCC and SCC was evaluated. **Methods :** Forty-seven BCCs and 43 SCCs were selected and microarrayed in paraffin blocks. Immunohistochemical analysis was performed with specific antibodies for bcl-2, bcl-6, p16 and p63. **Results :** p63 was found to be expressed in all BCCs and SCCs. Bcl-2 was exclusively expressed in BCCs (100%), but there was negative expression in SCCs, whereas bcl-6 was positively expressed in 18.2% of SCCs, and was negative in BCCs. In SCCs, p16 was expressed at high frequency (47.7%) than in BCCs (14.9%). The expression of p16 was correlated with the histologic grades of SCCs. **Conclusion :** The different patterns of bcl-2, bcl-6, p63 and p16 protein expression between BCCs and SCCs may represent the different histogenesis and morphologic features of two lesions.

Key Words : Carcinoma; Basal cell; Squamous cell; p63 protein; Proto-oncogene proteins bcl-2; Proto-oncogene protein bcl-6; Protein 16

The gene, *p63* is one of two recently identified genes that has been shown to encode several proteins whose structure and functions are similar to those of *p53*.[1] p63 protein is consistently expressed in the basal cells of stratified epithelia, and glandular structures.[2] In skin, p63 is considered as a reliable keratinocyte stem cell marker, and is used in the identification of these cells in the normal skin[3] and the tumors of epidermis and epidermal appendages.[4,5]

The *bcl-2* gene encodes 26 kDa protein that can prevent cells from programmed cell death conferring survival advantages and is implicated in follicular lymphoma.[6,7] The bcl-2 protein expression is mainly observed in cell populations with a long life and/or proliferating ability.[8,9] In skin, bcl-2-positive cells serve as reserve cells from which the squamous epithelium continuously renews itself.[9] As basal cell carcinoma (BCC) is thought to arise from basal keratinocytes, bcl-2 expression was detected in nearly all cases of BCC, whereas squamous cell carcinoma (SCC) was presumed to originate from suprabasal keratinocytes and was negative for bcl-2 expression.[9,10]

The *bcl-6* gene functions as a transcriptional repressor.[11] Its protein is essential for germinal center formation of lymph node, and is expressed in germinal center B cells and in their neoplastic counterparts.[12] However, bcl-6 protein expression has also been found in sites outside the lymphoid system.[13] Yoshida *et al*.[14] reported that bcl-6 may play a role in keratinocytes differentiation at terminal stage, and Kanazawa *et al*.[15] described a variable expression of bcl-6 in SCC, but none in BCC.

The gene, *p16* is encoded by the *CDKN2A* gene on chromosome 9q21. It binds to CDK4 and inhibits phosphorylation of the Rb protein, leading to arrest of cell cycle in G1 phase with subsequent suppression of cell proliferation. During the functional or structural loss of *p16*, the cells enter the phase of cell division.[16] About approximately 80% of malignant skin tumors have found to be associated with loss of *p16* gene.[17] However, there have been controversies on the issue of p16 protein expression. Tam *et al*.[16] described that some tumor cell lines showed no p16 protein expression, whereas others showed overexpression, compared to their normal counterparts. Recent studies have reported that invasive and noninvasive squamous cell carcinomas of the skin[18] and uterine cervix[19] were associated with overexpression

of p16 protein.

In the present study, two distinct and common malignant neoplasms of the skin, BCC and SCC were selected and the expression patterns of bcl-2, bcl-6, p63 and p16 were compared in order to evaluate their usefulness in differentiatial diagnosis of carcinomas.

MATERIALS AND METHODS

Tumor specimens

Forty-seven BCCs and 43 SCCs of the skin were retrospectively retrieved from the files of the Department of Pathology, Korea University Hospital. The clinical information was obtained from the file of the pathology requests. All pathological slides were reviewed and one appropriate paraffin block was selected from each case.

Construction of the tissue microarray

The representative tissue area from each case was taken by using 2.0 mm punch, and inserted into a recipient paraffin block to create a tissue microarray. Four micrometer-thick sections were cut from the completed array block and transferred to silanized glass slides.

Immunohistochemical staining

For p16, $p16^{INK4a}$ Research Kit (mtm Laberatories AG, Germany) was used, and the staining was performed according to the manufacture instruction. For p63, bcl-2 and bcl-6, DAKO LSAB Kit (DAKO A/S, Denmark) was used.

The 4 μm-thick tissue sections were deparaffinized and rehydrated. For *p16* staining, the slides were incubated at 95-99℃ for 40 min in epitope retrieval solution, and were then cooled at room temperature (RT) for 20 min. After soaking in washing buffer for 5 min, the slides were covered by peroxidase-blocking reagent, and were incubated at room temperature for 5 min. After gentle rinse, the slides were covered with anti-human $p16^{INK4A}$ antibody reagent (1:25) and were further incubated at RT for 30 min.

In the case of p63, bcl-2 and bcl-6, the endogenous peroxidase activity was eliminated by incubation with 3% H_2O_2 in methanol for 15 min, and the antigen retrieval was done by placing the slides in pressure cooker containing 2.0 L of 0.01 M sodium citrate buffer (pH 6.0) at 103 Kpa for 2 min. The slides were then incubated with primary monoclonal antibodies for p63 (4A4 antibody, 1:150, DAKO A/S, Denmark), bcl-2 (1:50, DAKO A/S, Denmark) or bcl-6 (1:10, DAKO A/S, Denmark) at RT for one hour. After incubation at RT for 30 min with biotinylated secondary antibody, the slides were incubated with streptavidin-peroxidase complex at RT for 30 min.

Immunostaining was developed by using 3,3′diaminobenzidine as chromogen. The slides were counterstained with Mayer's hematoxylin for 1 min.

Analysis of immunohistochemical staining

A semiquantitative assessment for p16, p63, bcl-2 and bcl-6 expression was carried out according to the following criteria: Negative (<5% positive staining of neoplastic cells), 1+ (5-25% positive staining of neoplastic cells), 2+ (26-50% positive staining of neoplastic cells), 3+ (51-75% positive staining of neoplastic cells), and 4+ (>75% positive staining of neoplastic cells).

Statistical analysis

Statistical analysis was performed by using Statview software (4.0, SAS Institute Inc., Cary, NC, USA). A level of $p<0.05$ was considered as being significant.

RESULTS

For the investigation of BCC, 22 male and 25 female patients were chosen. The primary sites of BCCs were on the face and scalp in 43 cases, on the trunk in 3 cases, and on the scrotum in one case. Histopathologically, 29 cases were solid type (61.7%), 7 cases were adenoid type (14.9%), and 4 cases were keratotic type (8.5%). Superficial type, morphea-like type and hair-follicular type were 4 (8.5%), 2 (4.3%) and one (2.1%), respectively (Table 1).

Table 1. Histologic types of basal cell carcinoma and histologic grades of squamous cell carcinoma

Histologic types of BCC	No. of cases (%)	Histologic grades of SCC	No. of cases (%)
Solid	29 (61.7)	Poorly differentiated	26 (59.1)
Adenoid	7 (14.9)	Moderately differentiated	12 (27.3)
Keratotic	4 (8.5)	Well differentiated	6 (13.6)
Superficial	4 (8.5)		
Morphea-like	2 (4.3)		
Hair-follicular	1 (2.1)		

BCC, Basal cell carcinoma; SCC, Squamous cell carcinoma.

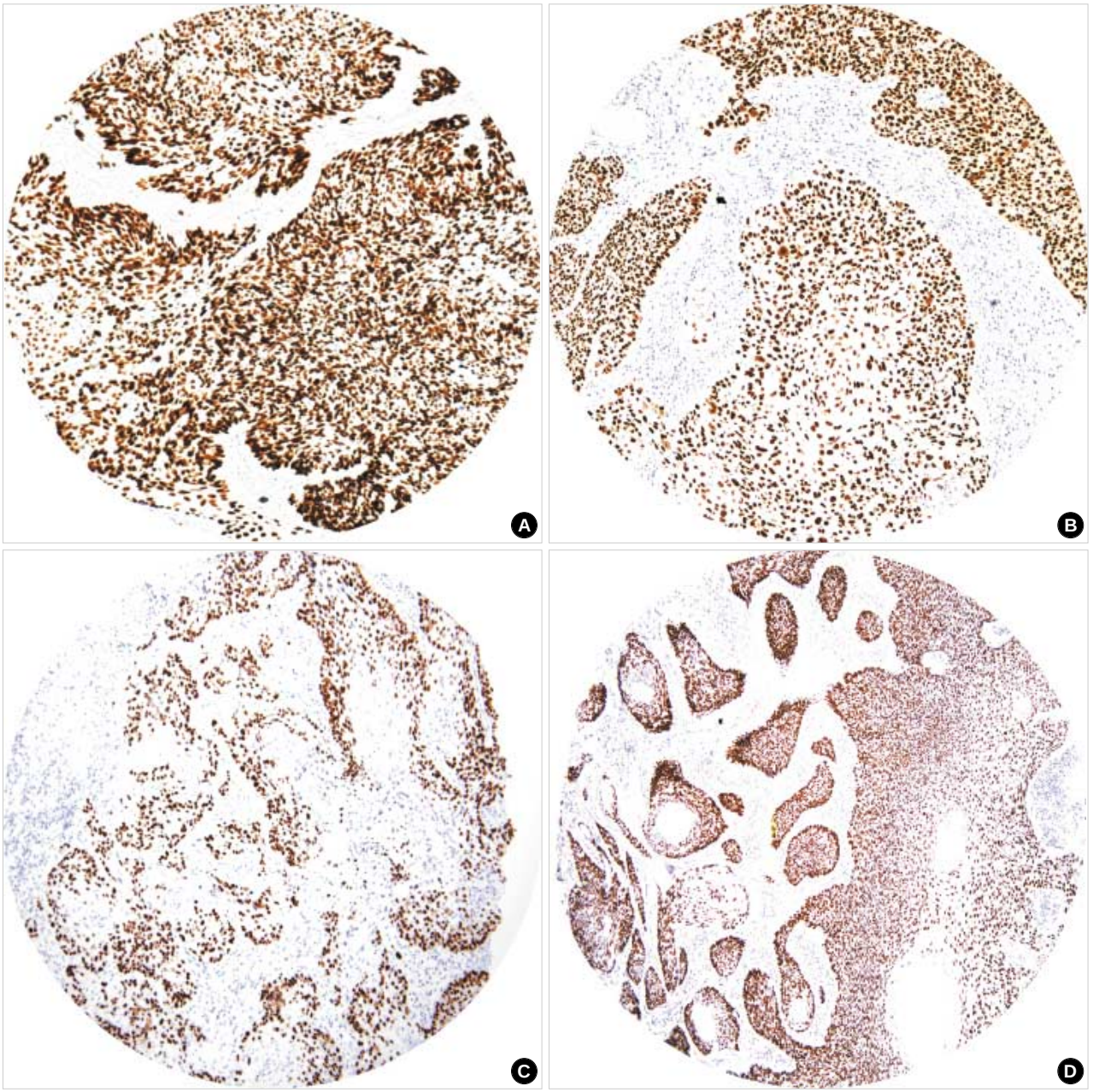

Fig. 1. The immunohistochemistry for p63 shows strong and diffuse nuclear staining in solid type basal cell carcinoma (A) and moderately differentiated squamous cell carcinoma (B). The central nests of squamous cells in keratotic basal cell carcinoma (C) and the keratinizing cells in well differentiated squamous cell carcinoma (D) are negative.

For the examination of SCC, 27 male and 16 female patients were investigated. The primary sites of SCCs were on the face and scalp in 25 cases, on the limbs in 11 cases, on the trunk in 5 cases and on the scrotum in two cases. Histopathologically, poorly differentiated type was 26 cases (59.1%), moderately differentiated type 12 cases (27.3%), and well differentiated type 6 cases (13.6%).

Expression of p63

In normal skin, p63 was consistently expressed in the nuclei of the epidermal basal cells, the cells of the germinative hairmatrix and the external root sheath of the hair follicles.

All BCC cases, regardless of the histologic types, showed 4+ positive staining for p63. In SCCs, the 4+ expression was seen in 23.1% of poorly differentiated and 33.3% of moderately dif-

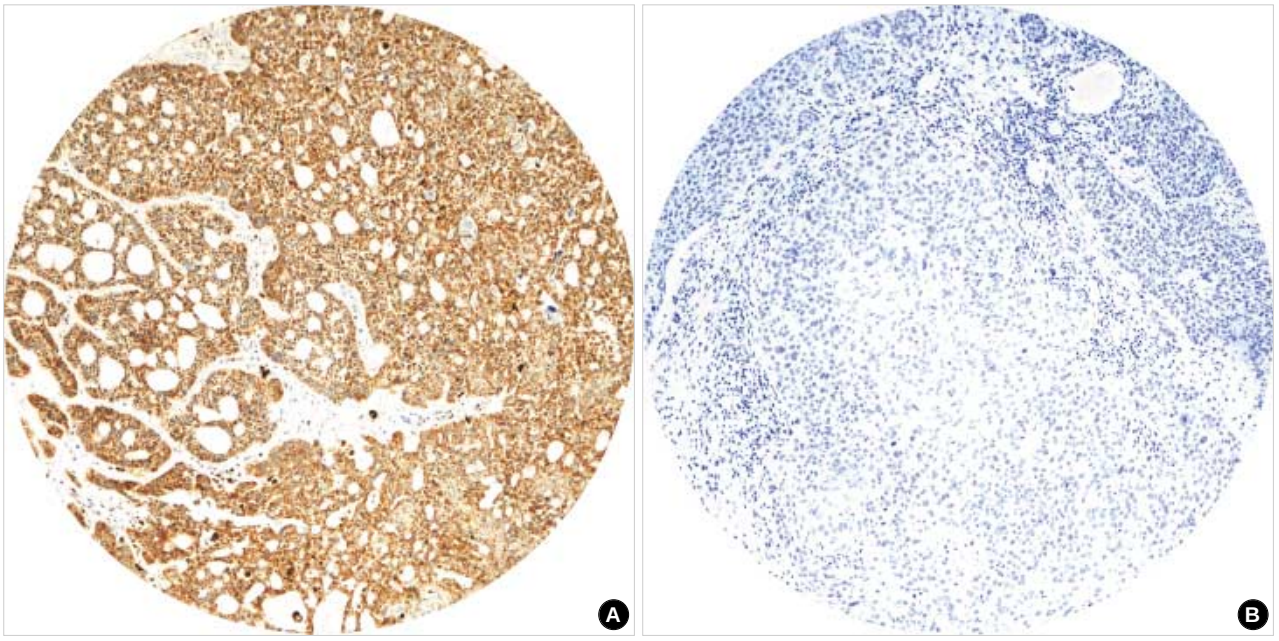

Fig. 2. The immunohistochemistry for bcl-2 shows strong and diffuse membrane and cytoplasmic staining in adenoid type basal cell carcinoma (A), but negative in squamous cell carcinoma (B).

Table 2. Expression of p63 in basal cell carcinoma and squamous cell carcinoma

	No. of p63 expression (%)					
	Total	-	1+	2+	3+	4+
BCC	47	0 (0)	0 (0)	0 (0)	0 (0)	47 (100)
Solid	29	0 (0)	0 (0)	0 (0)	0 (0)	29 (100)
Adenoid	7	0 (0)	0 (0)	0 (0)	0 (0)	7 (100)
Keratotic	4	0 (0)	0 (0)	0 (0)	0 (0)	4 (100)
Superficial	4	0 (0)	0 (0)	0 (0)	0 (0)	4 (100)
Morphea-like	2	0 (0)	0 (0)	0 (0)	0 (0)	2 (100)
Hair-follicular	1	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)
SCC	44	0 (0)	20 (45.5)	8 (18.2)	6 (13.6)	10 (22.7)
PD	26	0 (0)	13 (50.0)	4 (15.4)	3 (11.5)	6 (23.1)
MD	12	0 (0)	5 (41.7)	2 (16.7)	1 (8.3)	4 (33.3)
WD	6	0 (0)	2 (33.3)	2 (33.3)	2 (33.3)	0 (0)

PD, Poorly differentiated; MD, Moderately differentiated; WD, Well differentiated.
-: <5%, 1+: 5-25%, 2+: 26-50%, 3+: 51-75%, 4+: >75%.

Table 3. Expression of bcl-2 in basal cell carcinoma and squamous cell carcinoma

	No. of bcl-2 expression (%)					
	Total	-	1+	2+	3+	4+
BCC	47	0 (0)	14 (29.8)	12 (25.5)	12 (25.5)	9 (19.1)
Solid	29	0 (0)	10 (34.5)	6 (20.7)	8 (27.6)	5 (17.2)
Adenoid	7	0 (0)	1 (14.3)	2 (28.6)	2 (28.6)	2 (28.6)
Keratotic	4	0 (0)	1 (25.0)	1 (25.0)	1 (25.0)	1 (25.0)
Superficial	4	0 (0)	1 (25.0)	2 (50.0)	0 (0)	1 (25.0)
Morphea-like	2	0 (0)	0 (0)	1 (50.0)	1 (50.0)	0 (0)
Hair-follicular	1	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)
SCC	44	44 (100)	0 (0)	0 (0)	0 (0)	0 (0)
PD	26	26 (100)	0 (0)	0 (0)	0 (0)	0 (0)
MD	12	12 (100)	0 (0)	0 (0)	0 (0)	0 (0)
WD	6	6 (100)	0 (0)	0 (0)	0 (0)	0 (0)

ferentiated tumors, but none in well differentiated tumors. Compared to the expression in BCCs, SCCs exhibited a variable degree of staining pattern; 4+ in 22.7%, 2+ and 3+ in 31.8%, and 1+ in 45.5% of the cases (Table 2, Fig. 1). The expression of p63 was higher in BCCs than in SCCs ($p<0.05$).

Expression of bcl-2

In normal skin, the bcl-2 expression was confined to the basal keratinocytes that exhibited cytoplasmic staining with perinuclear enhancement. In BCCs, a higher than 2+ staining was found in 69.2% of the cases, and the remaining cases showed 1+ staining. There was no difference among the histologic types. In SCCs, none of the cases were positive for bcl-2 (Table 3, Fig. 2). The expression of bcl-2 was higher in BCCs than in SCCs ($p<0.05$).

Expression of bcl-6

In normal skin, the bcl-6 protein was shown to be intensely stained on the nuclei of the normal prickle cells, but none on the epidermal basal cells. In BCCs, the bcl-6 expression was restricted to the prickle cell layers of uninvolved area, but was totally

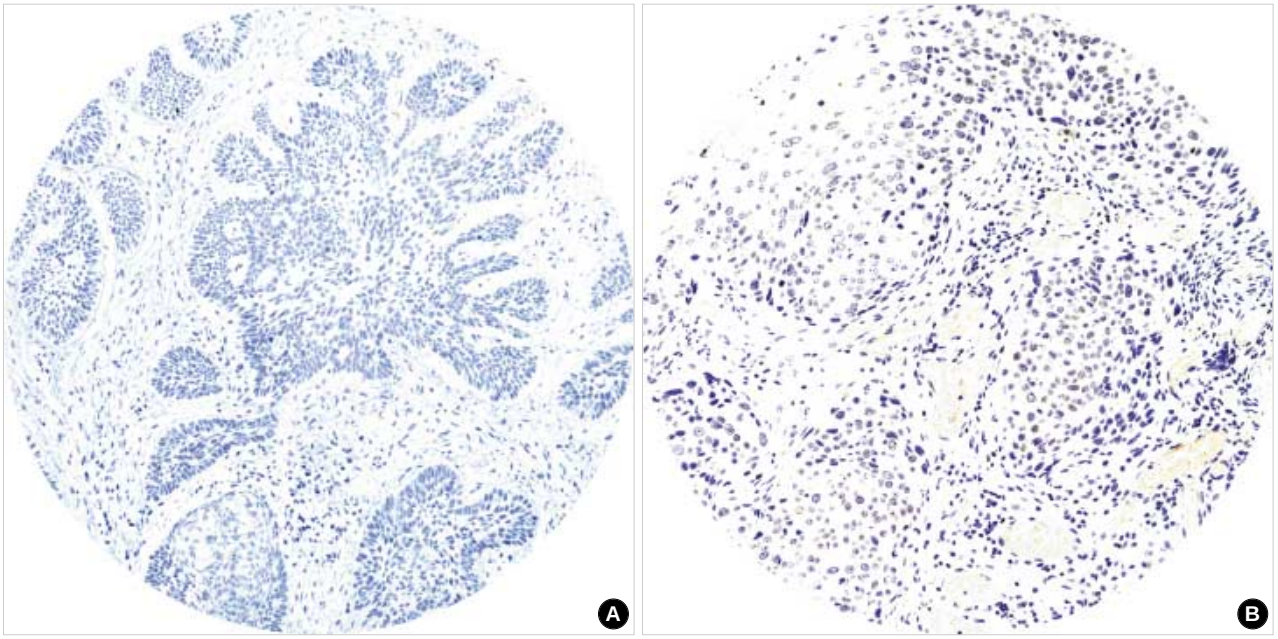

Fig. 3. The immunohistochemistry for bcl-6 was negative in basal cell carcinoma (A), but partly positive in squamous cell carcinoma (B).

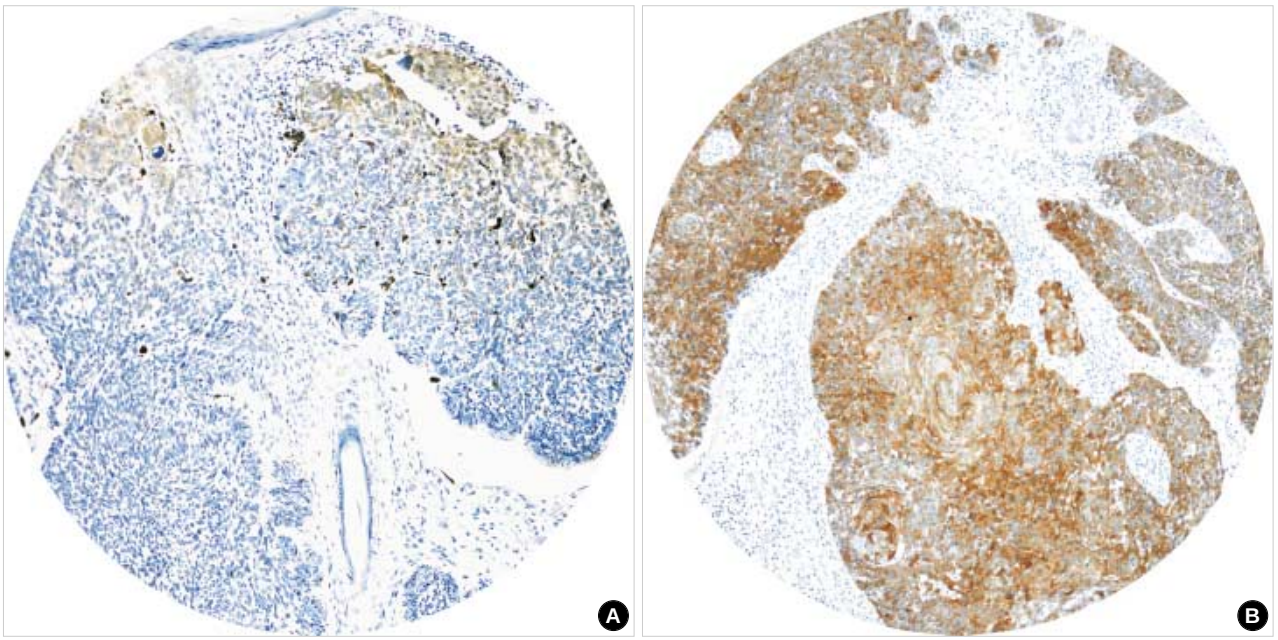

Fig. 4. The immunohistochemistry for p16 shows nuclear and cytoplasmic staining in some of tumor cells in basal cell carcinoma (A), but diffuse and strong positive in squamous cell carcinoma (B).

negative in the tumor cells. However, SCCs exhibited a 1+ nuclear staining in 18.2% of the cases (Table 4, Fig. 3). The expression of bcl-6 was higher in SCCs than in BCCs ($p<0.05$).

Expression of p16

In normal skin, the immunostaining for p16 was negative in squamous epithelium. In BCCs, 14.9% of the cases (solid, adenoid, keratotic and superficial types) showed 1+ staining on the nucleus and cytoplasm. SCCs exhibited a variable degree of staining; 4+ in 9.1%, 2+ and 3+ in 11.4%, and 1+ in 79.5% of cases. The expression in SCCs was increased according to the degree of differentiation; 33.3% of poorly differentiated tumors, 63.6% of moderately differentiated tumors and 83.3% of well differen-

Table 4. Expression of bcl-6 in basal cell carcinoma and squamous cell carcinoma

	No. of bcl-6 expression (%)					
	Total	-	1+	2+	3+	4+
BCC	47	47 (100)	0 (0)	0 (0)	0 (0)	0 (0)
Solid	29	29 (100)	0 (0)	0 (0)	0 (0)	0 (0)
Adenoid	7	7 (100)	0 (0)	0 (0)	0 (0)	0 (0)
Keratotic	4	4 (100)	0 (0)	0 (0)	0 (0)	0 (0)
Superficial		4 (100)	0 (0)	0 (0)	0 (0)	0 (0)
Morphea-like	2	2 (100)	0 (0)	0 (0)	0 (0)	0 (0)
Hair-follicular	1	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)
SCC	44	36 (81.8)	8 (18.2)	0 (0)	0 (0)	0 (0)
PD	26	20 (76.9)	6 (23.1)	0 (0)	0 (0)	0 (0)
MD	12	11 (91.7)	1 (8.3)	0 (0)	0 (0)	0 (0)
WD	6	5 (83.3)	1 (16.7)	0 (0)	0 (0)	0 (0)

Table 5. Expression of p16 in basal cell carcinoma and squamous cell carcinoma

	No. of p16 expression (%)					
	Total	-	1+	2+	3+	4+
BCC	47	40 (85.1)	7 (14.9)	0 (0)	0 (0)	0 (0)
Solid	29	26 (89.6)	3 (10.3)	0 (0)	0 (0)	0 (0)
Adenoid	7	5 (71.4)	2 (28.6)	0 (0)	0 (0)	0 (0)
Keratotic	4	3 (75)	1 (25)	0 (0)	0 (0)	0 (0)
Superficial	4	3 (75)	1 (25)	0 (0)	0 (0)	0 (0)
Morphea-like	2	2 (100)	0 (0)	0 (0)	0 (0)	0 (0)
Hair-follicular	1	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)
SCC	44	23 (52.3)	11 (25.0)	4 (9.1)	3 (6.8)	3 (6.8)
PD	26	18 (66.7)	4 (14.8)	2 (7.4)	2 (7.4)	1 (3.7)
MD	12	4 (36.4)	3 (27.3)	1 (9.1)	1 (9.1)	2 (18.2)
WD	6	1 (16.7)	4 (66.7)	1 (16.7)	0 (0)	0 (0)

tiated tumors ($p<0.05$) (Table 5, Fig. 4). The expression of p16 was higher in SCCs than in BCCs ($p<0.05$).

DISCUSSION

BCC and SCC are the most common malignant cutaneous neoplasms in humans. Histologically, BCCs lack precursor lesions and can be subdivided into a number of sub-types. Clinically, BCC is characterized by local invasion and contiguous spread. While reports of metastatic BCC exist in the literatures, it is widely recognized that metastasis of BCC is an extremely rare event, in contrast to SCC.[20] Histologically, SCC displays a number of variations from BCC, including a series of well-defined precursors, lack of well-demarcated tumor periphery and the presence of features of epidermal differentiation.[21] SCC is a biologically aggressive tumor and may metastasize at frequencies of 1-12.5%.[21] Following local invasion and tissue destruction, SCC commonly metastasizes to lymph nodes. In the present study, the expressions of p63, bcl-2, bcl-6 and p16 are examined in these two common cutaneous cancer types.

p63 gene has a similar structure with *p53* and encodes 6 isoforms.[1] Three TAp63 isoforms have N-terminal transactivation domain capable of transactivating *p53* target genes and inducing cell-cycle arrest, whereas three ΔNp63 isoforms lack the transactivation domain and block the function of p53 and TAp63.1,[22] In normal skin, the expression of p63 is gradually reduced from the basal cells to the terminally differentiated keratinocytes.[3] Among malignant tumors, the consistent expression of p63 has been reported in basal cell carcinoma of the skin, squamous cell carcinoma of the skin, uterine cervix, head and neck, and lung, and transitional cell carcinoma of urinary bladder, and is usually unexpressed in adenocarcinomas of the various organs.[2,23] In the present study, the expression of TAp63 was detected by immunohistochemistry using monoclonal 4A4 antibody. All BCCs showed strong and diffuse 4+ expression for p63. The entire keratinizing horny layers in keratotic types showed negative expression of p63. In contrast, SCCs showed variable expression of p63. The differentiated keratinocytes forming the pearls in well differentiated SCC did not show any p63 expression, but the less and poorly differentiated tumor cells showed strong and diffuse expression. These findings were similar with those of the previous studies.[4,5] The consistent expression of p63 in BCCs and SCCs indicates that p63 protein is important in tumorigenesis of epithelial cells of the skin. Further studies evaluating differential expression of p63 isoforms should be carried out in order to clarify the role of the different p63 isoforms in development of skin cancers.

The expression of bcl-2 protein in skin tumors was first noted in 1994 by Smoller *et al*.[24] They described that bcl-2 expression reliably distinguished trichoepitheliomas from basal cell carcinomas. Except the result of Nakagawa *et al*.[25] other studies have reported that nearly 100% of BCCs and malignant melanomas expressed bcl-2, whereas SCCs showed no expression or small numbers of positive cells.[10,26] In this study, bcl-2 was variably expressed in all BCCs, whereas none of SCCs expressed bcl-2. The overexpression of bcl-2 protein in follicular lymphomas is the result of chromosomal translocation. However, the protein expression can occur without any chromosomal rearrangement.[27] Morales-Ducret *et al*.[10] evaluated chromosomal translocation in one of BCCs with bcl-2 overexpression, however, no rearranged band was detected.

The overexpression of bcl-2 in BCCs may simply represent its expression in normal counterpart, basal keratinocytes, rather than dysregulation or abnormal expression. The lack of bcl-2 expres-

sion in SCC suggested that most of tumor cells may originate from the suprabasal keratinocytes which show negative bcl-2 expression.[9,10]

It has been reported that the expression of bcl-2 was higher in indolent superficial and circumscribed subtypes than aggressive tumors.[28] However, in the present cases, there was no difference in bcl-2 expression among the histologic subtypes.

Bcl-6 is exceptionally associated with the pathogenesis of some diffuse large B cell lymphomas of germinal center origin.[12] In this study, bcl-6 protein was intensely expressed in the nuclei of the upper layer cells in the normal epidermis, and exhibited variable expression in SCCs, but none of BCCs showed bcl-6 expression. The expression of bcl-6 in transitional cell carcinoma of urinary bladder is associated with high histologic grade.[13] In this study, there was no difference in bcl-6 expression based on the degree of differentiation of SCC.

Deletion, mutation, or hypermethylation of *p16* gene has been found in various types of human cancers.[17] Among skin cancers, mutation or deletion of *p16* gene has been detected in malignant melanoma and SCC, and has been associated with loss of mRNA and protein expression.[17,29] However, p16 protein expression was not negative but was overexpressed in some tumors, when compared to the normal tissues,[16] and was true in SCC of head and neck, and uterine cervix.[19] Overexpression of p16 in uterine cervical cancers is thought to be related with the inactivation of Rb by E7 of high-risk HPV, but it does not seem to operate in cutaneous cancers. Recent studies[18,30] on premalignant and malignant squamous lesions of the skin showed a progressively increased expression of p16 protein from actinic keratosis or low grade intraepithelial lesions to high grade lesion and invasive cancer. In this study, p16 expression was stronger in SCCs than in BCCs, and the positive rate was higher in well differentiated tumor than moderate and poorly differentiated tumors. This result could not be compared with other studies, as there has been neither any data on BCC nor any studies on the grade of SCC.

In summary, bcl-2 was exclusively restricted to the BCCs, p63 was expressed in both BCCs and SCCs, bcl-6 was expressed in SCCs, but not in BCCs, and p16 was more frequently expressed in SCCs than BCCs. The differences in the pattern of bcl-2, bcl-6, p63 and p16 protein expression between BCCs and SCCs may represent the different origin of the tumor cells as well as different histologic features of two lesions. Further molecular and genetic studies should be done to solve the problem.

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