Immunohistochemical nuclear staining for p53, PCNA, Ki-67 and bcl-2 in different histologic variants of basal cell carcinoma

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Abstract
Background: Basal cell carcinoma is the most common form of human cancer. Increased expression of p53 has been found in the majority of basal cell carcinomas (BCCs); however, UV-light-induced signature mutations are present in only about 50% of cases. Increased nuclear staining with an immunohistochemical marker of proliferation and apoptosis has been correlated with aggressive behavior in BCC.
Objective: Our purpose was to correlate markers expression of apoptosis (p53 and bcl-2) and cell proliferation (Ki-67 and PCNA) with histological indicators of tumor severity.
Methods: We used immunohistochemical stains for p53, PCNA, and Ki-67, in superficial, nodular and sclerosing BCC, to determine whether the staining patterns differ in these different histologic variants of BCC.
Results: Bcl-2 expression was significant in basal cell carcinomas said to be aggressive (morpheaform and nodular types). Of the studied tumors, 66.7% (n=14) strongly expressed p53. Our results show a greater expression of Ki-67 in nodular and superficial basal cell carcinoma. PCNA showed a strong expression in all types of tumors.
Conclusion: Studies employing molecular and genetic biology techniques, associated with histomorphology, lead to the identification of risk factors in the development of more recurring and aggressive lesions.

Keywords: carcinoma, basal cell, cell cycle proteins, immunohistochemistry, skin neoplasms, tumor markers.

Introduction
Basal cell carcinoma (BCC) is one of the most common cancers known to man. While having a negligible rate of mortality and metastatic activity, they nonetheless are progressively enlarge and locally invasive tumors. When one or more cutaneous tumors are present in an individual patient, multiple biopsy samples can be easily obtained for studies. In recent years, the kinetics of cell proliferation in normal and tumor tissues has been actively studied for pathophysiological and chemotherapeutic clues [1].

Mutations in p53 may occur early or late in the stepwise development of epithelial malignancy. The chronic exposure to sunlight has been found to be responsible for accumulation of p53 mutation in cutaneous carcinogenesis [2–4]. Previous studies concluded that detectable p53 protein is a common occurrence in malignant epithelial lesions, but it does not correlate with the malignant phenotype or with metastatic p53 protein in basal cell carcinomas. However, the relationship between age of patients and expression of p53 protein in normal epithelia adjacent to carcinomas seems to support the role of the p53 mutation in early BCC progression [5].

Bcl-2, the well-known anti-apoptotic gene, cloned more than a decade ago, promotes cell viability without promoting cell proliferation. With few exceptions, high bcl-2 protein expression is associated with a favorable outcome in epithelial tumors. Bcl-2 protein expression as a predictor of BCC aggressiveness is poorly documented in the English-language literature [6].

Proliferating cell nuclear antigen (PCNA) is a nuclear protein that is expressed in G1-M phases of the cell cycle, but is maximally expressed in late GDS phases [7]. Previous immunohistochemical studies have shown increased nuclear PCNA staining in clinically and histologically aggressive BCC [8–10].

Another immunohistochemical marker of cellular proliferation is Ki-67, a non-histone protein located predominantly in the nucleolus. During mitosis, Ki-67 is associated with surfaces of condensed chromatin and the chromosomes, and after cell division, it is located in the nucleoplasm before localizing in the nucleoli [11]. Although Ki-67 is expressed throughout late G1-M phases of the cell cycle, it is maximally expressed at the G2-M interface. In normal skin, expression of PCNA in the epidermis is generally limited to some cells of the basal cell layer and occasionally in the suprabasal cell layer. Variable intensity levels of Ki-67 have been reported to be expressed more diffusely within the basal layers of the epidermis [12–15]. G1 and G2 cell blocks, which may occur with DNA damage, may have different effects on the presence of and intensity of expression of PCNA and Ki-67, because the maximal expression of these antigens occurs at different points in the cell cycle [16–18]. Expression of PCNA is also upregulated during DNA repair [19].
We performed immunohistochemical stains for p53, bcl-2, PCNA, and Ki-67 in different histopathologic variants of BCC including superficial, nodular and sclerosing BCC.

Materials and Methods

Twenty-one biopsies were performed in 19 patients. Seven samples of the nodular, morpheaform and superficial types of basal cell carcinoma were studied. Hematoxylin-eosin stained sections from all specimens were reviewed.

Immunostaining was performed on formalin-fixed in 10% buffered formalin and paraffin-embedded tissues based on an Avidin–Biotin–peroxidase complex technique [20, 21]. Sectioned tissue (3 µm thick) was deparaffinized with xylene and rehydrated through descending strengths of alcohol.

Endogenous peroxidase activity was blocked by incubating specimens in 2% hydrogen peroxide for 5 minutes. Before blocking for non-specific protein binding with normal goat serum and bovine serum albumin an antigen retrieval step was performed by boiling the sections for 15 minutes in 10 mmol/L citrate buffer at pH 6. The slides were then incubated overnight at 4°C in a humidified chamber with a primary antibody PCNA (DAKO PCNA, PC10) (1:100), p53 (DAKO p53, DO-7) (1:100), bcl-2 (DAKO bcl-2, D5) (1:100) and Ki-67 (DAKO Ki-67, MM-1) (1:100) (Table 1).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Dilution</th>
<th>Incubation period [hrs.]</th>
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<tr>
<td>Bcl-2</td>
<td>D5</td>
<td>1:100</td>
<td>18</td>
</tr>
<tr>
<td>p53</td>
<td>DO-7</td>
<td>1:100</td>
<td></td>
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<tr>
<td>Ki-67</td>
<td>MM-1</td>
<td>1:200</td>
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<tr>
<td>PCNA</td>
<td>PC-10</td>
<td>1:400</td>
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After rinsing with a phosphate-buffered saline solution for 15 minutes, the biotinylated secondary antibody was applied for 30 minutes at room temperature. After an incubation step with Avidin–Biotin–horseradish peroxidase conjugate for 30 minutes, sections were stained with 0.04% 3,3’-diaminobenzidine tetrahydrochloride and counterstained with Hematoxylin.

For each of the 30 BCCs, five high-power (×400) fields of the tumor with the highest density of stained nuclei were chosen and the percentage of PCNA, Ki-67, and p53-positive cells/total tumor cell population were scored as follows: 0–5% positive cells = negative, 6–25% = 1+, 26–50% = 2+, 51–75% = 3+, >75% = 4+. “High” labeling encompassed 3+ or 4+ labeling while “low” labeling referred to 1 + or 2 + labeling.

The intensity and distribution of nuclear staining were also graded. Intensity of nuclear staining was determined by the intensity of staining of the majority of the positive cells. Cells with light nuclear staining were not counted. To be judged as moderately intense staining, the staining had to be clearly more intense than any background staining on the slide with sharp demarcation of the nucleus.

Results

We performed immunohistochemical stains for p53, bcl-2, PCNA, and Ki-67 in superficial, nodular and sclerosing variants of BCC. Hematoxylin–Eosin stained sections from all specimens were reviewed (Figures 1–3).
the remaining superficial BCCs, the PCNA and Ki-67 nuclear staining was approximately equivalent (Figure 4).

The nodular BCCs demonstrated moderately intense staining for p53, PCNA, and Ki-67 (Figure 5).

The percentage of cells that demonstrated positive nuclear staining for PCNA was greater than Ki-67; however, the percentage of PCNA-positive tumor cells was always less than 10%. Some areas of peripheral accentuation were seen with p53 and PCNA in the majority of nodular BCCs. The superficial and nodular BCCs are indolent slow-growing tumors with high bcl-2 labeling (Figure 6).

The sclerosing BCCs showed more intense and higher numbers of positive cells for both p53 and the proliferation antigens. In the sclerosing and infiltrative BCCs, there was peripheral accentuation of both p53 and PCNA in the majority of the tumors. In sclerosing BCC the PCNA staining was greater than that of Ki-67 (Figure 6). The sclerosing BCCs are aggressive, infiltrative, desmoplastic tumors with low bcl-2 labeling (Figure 7).

Overall, in the sclerosing type of BCC, the staining was more intense, and more nuclei throughout the tumors showed a positive reaction than in the nodular and superficial BCCs. There was more staining for PCNA than Ki-67 in the sclerosing and infiltrating types of BCC and the nodular BCC. The superficial BCC showed more variability in staining than other types of BCC. In addition, in some of the superficial BCCs there was greater staining for Ki-67 than PCNA.

**Discussion**

Bcl-2 immunoreactivity in basal cell carcinomas (BCCs) is contradictory, with 67–100% immunopositivity being reported. Although BCCs are traditionally regarded as low-grade, indolent tumors, aggressive BCCs are mutilative, locally destructive tumors that often recur [6]. The differential bcl-2 expression in the various clinicopathological subtypes of BCCs suggests that, despite the common derivation of these tumors from a primitive basaloid stem cell and a limited potential for metastasis, they form a heterogeneous group of tumors that differ markedly in histologic and biological behavior. While the superficial and nodular BCCs are indolent slow-growing tumors with high bcl-2 labeling, the sclerosing BCCs are infiltrative, desmoplastic tumors with low bcl-2 labeling.

Wild-type (wt) p53, which has been dubbed the guardian of the genome, mediates arrest of the cell cycle in the G1 phase after sublethal DNA damage. 18 p53 is a transcription factor with a sequence specific DNA-binding domain [22]. Conserved regions of this core domain (“hotspots”) are the sites of the majority of p53 mutations that have been identified in human tumors [23]. Polyclonal and monoclonal antibodies for wt and mutant p53s are available [24]. The low levels of wt p53
protein in normal tissue usually cannot be detected with immunohistochemical stains with some exceptions. Wt p53 increases after DNA damage. After sun exposure, wt p53 is increased in keratinocytes of normal skin [24, 25]. Up-regulation of wt p53 in skin after UV light exposure represents the normal mechanism for induction of a G1 block to allow apoptotic cell death or repair of DNA damage. The low levels of wt p53 in normal skin reflect the rapid degradation of wt p53 [17, 22, 23]. A large percentage of the mutant p53s appear more stable than wt p53, and these accumulate in cells. Antibodies, such as the one we used, that bind to an epitope in a conserved region near the N-terminus of p53, outside the conserved region near the N-terminus of p53, outside such as the one we used, that bind to an epitope in a conserved region near the N-terminus of p53, outside such as the one we used, that bind to an epitope in a conserved region near the N-terminus of p53, outside such as the one we used, can detect both wt p53 and the majority of mutant p53s [22, 23, 26]. Because mutant p53 is usually more stable than wt p53, increased immunohistochemical staining with this antibody usually occurs because of mutant p53. This reflects loss of function of p53. However, no antibody will detect all mutants, and no antibody will detect cases in which the mutations, including deletions in the genome, result in the failure to produce a protein. Thus, the tumors that were p53 negative may have had a p53 mutation. Loss of function of p53, with negative immunohistochemical staining, may also occur with increased degradation of p53. This is caused by some viral proteins as well as by up-regulation of cellular proteins such as mdm-2 [22, 24]. The significance of loss of function of p53 may depend on whether loss of function occurs early or late in malignant transformation. In SCC and BCC from sun-exposed skin, mutant p53 appears to be an early event [27]. The presence of a p53 mutation in these epithelial lesions may be significant in the oncogenic process itself, by decreasing the cells’ ability to deal with DNA damage. As expected almost all BCCs studied showed increased nuclear immunostaining for p53, except for superficial BCC, in which half the tumors were p53-negative.

If p53 mutations are less common in superficial BCC, a decrease in function of p53 could still be present if there was an increased degradation of p53 by either an exogenous (i.e., viral) or endogenous (i.e., mdm-2) protein.

However, if p53 mutations are present in superficial BCC, our finding could be explained if these mutations were in areas of genome that resulted in a protein that was not detected by the antibody used, or if the mutation resulted in no p53 protein production. Our findings also suggest that if a p53 mutation is present in an adjacent AK, it may not be present or may not be the same mutation that is present in the adjacent BCC.

In p53-negative superficial BCC, the nuclear staining for Ki-67 and PCNA was approximately equivalent, whereas nuclear staining for Ki-67 was greater than the PCNA in three superficial BCCs that did show increased p53 nuclear staining.

In all other BCC variants, the increased nuclear staining for PCNA was greater than or approximately equivalent to the staining for Ki-67. The increase in PCNA was greatest in the cells that expressed p53 most intensely and diffusely. Up-regulation of wt p53 in some superficial BCCs, is consistent with a greater increase in Ki-67, whereas the presence of mutant p53 should lead to a greater increase in PCNA as was seen in all other BCC variants. The percentage of Ki-67+ cells in all classes of BCC varied less and were fewer in number than in one previous study [11].

DNA damage resulting from UV radiation leads primarily to G1 blocks with up-regulation of wt p53 [25–28]. Cells that have not entered S phase are blocked from progression through the cell cycle, and most undergo apoptotic cell death [25, 29, 30]. Cells in the S-phase at the time of DNA damage progress through to G2/M phase [25]. Thus up-regulation of wt p53, in the absence of a G2 block, should lead to a greater increase in Ki-67 than PCNA. Increased nuclear staining for mutant p53 reflects a loss of function of p53. This severely limits the ability of the cell to induce a G1 block as a response to DNA damage. Although loss of function of p53 compromises the cell’s ability to deal with DNA damage, some DNA repair will continue as the cell proceeds through the cell cycle and, in some instances, G2 blocks may be important in repair of the DNA damage preceding mitosis. PCNA is up-regulated during DNA repair, and PCNA is expressed in highest levels in the late G1–S phase. Progression to late G1–S phase can no longer be blocked by mutant p53 with loss of function of this protein [12, 19]. Thus expression of PCNA should be greater than Ki-67 in tumors with mutant p53. Another factor that may increase the number as well as the intensity of the overall PCNA staining in BCC is that the duration of the S-phase of a BCC tumor cell is about twice that of basal keratinocytes of the epidermis [1, 31].

**Conclusions**

The findings allow us to conclude that bcl-2 and p53 show a tendency to indicate the severity of basal cell carcinoma. In contrast, Ki-67, due to its variable behavior, cannot be considered a marker of severity. Also, PCNA was not a good marker of cell proliferation.

**References**


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