Immunohistochemical evaluation of proliferative activity (Ki-67 index) in different histological types of cutaneous basal cell carcinoma

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Abstract: Evaluation of tumor cell proliferation status belongs to the basic prognostic indicators in a routine biopsy report. In cutaneous basal cell carcinoma (BCC), however, there are discrepancies about a true prognostic significance of this histopathological parameter. The aim of this study was to assess a proliferative activity (Ki-67 index) in BCCs of the skin. Biopsy specimens from 80 cutaneous BCCs (63 primary, 17 recurrent) of different histological types from 75 subjects (34 men, 41 women) were enrolled into this study. All samples were immunohistochemically stained by antibody against Ki-67 antigen (DAKO, clone MIB-1, dilution 1:100). For the statistical analysis, χ² test was employed. We found a striking percentage variability of nuclear Ki-67 expression in individual tumors (range 2–70%). Mean value of Ki-67 index was 27.4% (in primary tumors 28.1%, in recurrent lesions 25.6%). The highest Ki-67 expression occurred in infiltrative BCCs (average 38.1%), morpheaform BCCs (average 37.0%), and superficial BCCs (average 35.7%), the lowest expression was recorded in nodular BCCs (average 21.7%) and BCCs with adnexal (trichoepithelial) differentiation (18.6%). There were not persuasive and statistically significant quantitative differences in proliferation activity of tumor cells between the individual histological BCC types, as well as between primary and recurrent lesions. A distribution of Ki-67 positive cells in tumor nests was mostly irregular and areas with a high number of Ki-67 labeled cells often occurred adjacent to areas with a lower number of cells expressing this marker. Because of a marked Ki-67 staining variability, we can conclude that the simple quantification of BCC proliferation activity alone may not be sufficient for the prediction of further biological behavior, evolution and clinical outcome of this malignancy.

Key words: basal cell carcinoma; Ki-67; proliferation; growth fraction.

Abbreviations: BCC, basal cell carcinoma; pBCC, primary basal cell carcinoma; rBCC, recurrent basal cell carcinoma; PCNA, proliferating cell nuclear antigen.

Introduction

Basal cell carcinoma (BCC) constitutes approximately 70–80% of all malignant skin tumors and currently is the most common malignancy in human population (Tilli et al. 2005; Crowson 2006; Correa et al. 2009). In fact, this cancer represents a spectrum of several tumor subtypes with a variable histomorphological picture and biological behavior. In contrast to other cancers, it generally pursues a favorable clinical course, growing slowly and expanding only locally (Tilli et al. 2005; Crowson 2006; Correa et al. 2009). However, some cases may show ab initio an aggressive behavior, rapidly infiltrating deeper tissue structure, recurring after treatment, and sometimes (although very rarely) giving rise to metastatic spread (Tilli et al. 2005; Crowson 2006; Correa et al. 2009). Based on different biological behavior and prognosis we distinct 2 BCC subgroups: indolent variants (superficial and nodular type), and aggressive variants (infiltrative sclerosing and non-sclerosing type, micronodular BCC and metatypical carcinoma) (Tilli et al. 2005; Crowson 2006).

To date, there is an increasing demand on identification of those tumors, that are prognostically more unfavorable, and whose impact on the overall health status of patients is more serious. Thus, it is an attempt to identify risk factors for the development of more aggressive lesions, with greater potential for recurrence and metastases. Recent advances in molecular pathology and biology lead to the identification of various biomarkers in tumor tissue, that significantly participate in carcinogenesis, and whose detection is of great importance in the prediction of further clinical behavior of cancer (Correa et al. 2009; Bartoš et al. 2011).

A high degree of cell proliferation generally correlates with rapid tumor growth, lower degree of differentiation and hence, more aggressive clinical and biological behavior. The proliferative activity (growth frac-
tion) of cancers is usually detected immunohistochemically by antibodies against Ki-67 antigen, or proliferating cell nuclear antigen (PCNA). Ki-67 antigen is a non-histone nuclear protein expressed during active phases of cell cycle (G1, S, G2 and mitotic phase), being absent in “resting” (G0) phase (Scholzen & Gerdes 2000). Under normal conditions, this protein resides predominantly in the nucleolus. During mitosis, it 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 (Scholzen & Gerdes 2000). Immunohistochemical assessment of nuclear Ki-67 expression (Ki-67 index) in neoplastic cells allows a quantitative measure of their proliferation status, and it belongs to the basic prognostic indicators in a routine histopathological report (Scholzen & Gerdes 2000). In addition, a percentage of Ki-67 immunoreactivity also serves as one of the cut-off criteria for malignancy in particular neoplasms (Yokoi et al. 2005; De Wailly et al. 2012). In cutaneous BCC, however, there are some discrepancies about a true prognostic significance of the proliferative activity because various studies (Horlock et al. 1998; Cernea et al. 2005; Misiuk Hojlo et al. 2005; Chuprov 2008; Correa et al. 2009; Selim et al. 2009, Mateouin et al. 2011) have provided conflicting results. The aim of this study was to analyze the proliferation status of tumor cells, using immunohistochemical evaluation of Ki-67 antigen, in the biopsy samples of BCCs of the skin.

Material and methods

Biopsy samples from 80 retrospectively selected cases of cutaneous BCCs (63 primary, 17 recurrent) of different histological types from 75 subjects (34 men, 41 women) were enrolled into this study. Age of the patients varied from 41 to 97 years (mean age 69.2 years). All patients were treated at the clinical departments of the Faculty Hospital in Zilina (Slovakia) and all biopsy samples were histopathologically investigated at the Department of Pathology in Faculty Hospital in Zilina between December 2009 – December 2011. During this 2-year period, a total of 125 cases of microscopically verified BCCs of the skin were diagnosed at our institution. For the purpose of this study, we aimed to retrospectively select a set of tumors consisted of all histomorphological BCC types from different anatomical locations, as well as of both primary (pBCC) and recurrent (rBCC) lesions. Only samples with enough tumor tissue in the paraffin-embedded blocks to harvest representative slides for immunohistochemistry were chosen. Topographical distribution of the tumors was as follows: eyelids and periorcular regions (n = 21), nose and paranasal regions (n = 12), back (n = 10), auricles and periauricular regions (n = 8), cheeks (n = 9), forehead (n = 6), scalp (n = 4), neck (n = 3), upper extremity (n = 3), lower extremity (n = 3), and mandibular region (n = 1). Individual specimens of skin tissue were clinically obtained by total surgical excision, probatory (partial) incision, and in a case of huge destructive lesion on the scalp by partial craniectomy.

All biopsy samples were routinely fixed in buffered formalin, embedded in paraffin blocks, stained with hematoxylin and eosin, and slides were finally reviewed by pathologists in the light microscope. In cases of BCCs with features of specific (adnexal) differentiation, in addition to standard hematoxylin and eosin we also used some special histochemical staining methods (alcan blue, PAS, Masson, van Gieson) for better microscopic visualisation of tissue structure. Evaluation of proliferative activity (Ki-67 index) was performed by immunohistochemical staining using antibody against Ki-67 antigen. Immunostaining was made on formalin-fixed and paraffin-embedded tissue based on an avidin-biotin-peroxidase complex technique. Sectioned tissue (4 µm thick) was deparaffinized in xylene and rehydrated at decreasing ethanol concentrations. Endogeneous peroxidase activity was blocked by incubating specimens in hydrogen peroxide, and slides were then incubated overnight in a chamber with a primary monoclonal antibody against Ki-67 (DAKO, clone MIB-1, dilution 1:100). Immunoreactivity was considered positive, when evident staining of the neoplastic cells nuclei was found. Tumor cells with only focal or discontinual nuclear labeling were not counted. For each of the 80 BCCs, Ki-67 index was classified as number of Ki-67 positive tumor cells divided by the total number of neoplastic cells population counted and expressed as a percentage. Semi-quantitative grading of immunoreactivity was scored according to Lee et al. (2000) as follows: grade 0 (negative staining), grade +/- (1–5% of positive tumor cells), grade 1+ (6–25% of positive tumor cells), grade 2+ (26–50% of positive tumor cells), and grade 3+ (more than 50% of positive tumor cells). For the statistical analysis, chi-square test was employed and p value < 0.05 was considered to indicate statistical significance.

Results

In general, we found a striking percentage variability of nuclear Ki-67 expression in individual tumors (in primary lesions between 2–70%, in recurrent lesions between 5–60%). There was no case of BCC with absolutely negative Ki-67 staining. The mean value of Ki-67 index of all 80 cancers assessed was 27.4%.

As for histological type, the highest Ki-67 expression occurred in infiltrative BCCs (average 38.1%), morpheiform BCCs (average 37.0%), and superficial BCCs (average 35.7%), the lowest expression was recorded in nodular BCCs (average 21.7%) and BCCs with adnexal (trichoepithelial) differentiation (18.6%). In spite of these different numbers, the χ² analysis did not confirm a significant correlation between the individual histological tumor types and the percentage of Ki-67 labeling. In addition, we did not show notable percentage differences in the mean value of Ki-67 index between primary (average 28.1%) and recurrent (average 25.6%) BCCs. Considering semi-quantitative evaluation of Ki-67 immunoreactivity, grade 1+ occurred the most frequently (n = 32, 40.0%), followed by grade 2+ (n = 26, 32.6%), grade +/- (n = 13, 16.2%), and finally grade 3+ (n = 9, 11.2%). When we compared a set of both, primary and relapsing tumors, we have demonstrated only small distinction between them without statistical significance (p = 0.75). The results of all expression values are summarized in Tables 1 and 2.

A spatial distribution of Ki-67 positive cells in tumor nests was mostly irregular and areas with a high number of cells expressing Ki-67 antigen often occurred...
Table 1. Summary of the mean percentage, range and semi-quantitative grades of Ki-67 immunoreactivity in the individual histological types of all 80 BCCs investigated.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Histological BCC type</th>
<th>N</th>
<th>Mean (%)</th>
<th>Range (%)</th>
<th>+/– (N)</th>
<th>1+ (N)</th>
<th>2+ (N)</th>
<th>3+ (N)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infiltrative</td>
<td>8</td>
<td>38.1</td>
<td>25–70</td>
<td>0</td>
<td>2</td>
<td>5</td>
<td>1</td>
<td>p = 0.21</td>
</tr>
<tr>
<td>Morpheaform</td>
<td>5</td>
<td>37.0</td>
<td>20–70</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>p = 0.72</td>
</tr>
<tr>
<td>Superficial</td>
<td>7</td>
<td>35.7</td>
<td>15–70</td>
<td>0</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>p = 0.20</td>
</tr>
<tr>
<td>Nodular-infiltrative</td>
<td>16</td>
<td>31.8</td>
<td>5–70</td>
<td>3</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>p = 0.24</td>
</tr>
<tr>
<td>Metatypical carcinoma</td>
<td>4</td>
<td>31.3</td>
<td>25–40</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>p = 0.42</td>
</tr>
<tr>
<td>Superficial-nodular BCC\textsuperscript{b}</td>
<td>3</td>
<td>22.3</td>
<td>15–35</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>p = 0.7</td>
</tr>
<tr>
<td>Nodular</td>
<td>23</td>
<td>21.7</td>
<td>2–70</td>
<td>6</td>
<td>10</td>
<td>6</td>
<td>1</td>
<td>p = 0.28</td>
</tr>
<tr>
<td>BCC with adnexal differentiation</td>
<td>14</td>
<td>18.6</td>
<td>2–45</td>
<td>4</td>
<td>6</td>
<td>4</td>
<td>0</td>
<td>p = 0.3</td>
</tr>
</tbody>
</table>

Total                           | 80 | 27.4     | 2–70      | 13 (16.2%) | 32 (40%) | 26 (32.6%) | 9 (11.2%) |

\textsuperscript{a} BCC types are arranged descendingly in relation to the mean percentage of Ki-67 positivity (N – number of cases; +/–, 1+, 2+, 3+ – individual grades of Ki-67 positivity; statistical significance at \( p < 0.05 \)).

\textsuperscript{b} With infiltrative component.

Table 2. Comparison of the mean percentage, range and semi-quantitative grades of immunohistochemical Ki-67 expression in relation to primary and recurrent BCCs.\textsuperscript{a}

<table>
<thead>
<tr>
<th>BCC</th>
<th>N</th>
<th>Mean (%)</th>
<th>Range (%)</th>
<th>+/– (N)</th>
<th>1+ (N)</th>
<th>2+ (N)</th>
<th>3+ (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary BCC</td>
<td>63</td>
<td>28.1</td>
<td>2–70</td>
<td>10 (15.8%)</td>
<td>26 (41.3%)</td>
<td>19 (30.2%)</td>
<td>8 (12.7%)</td>
</tr>
<tr>
<td>Recurrent BCC</td>
<td>17</td>
<td>25.6</td>
<td>5–60</td>
<td>3 (17.6%)</td>
<td>6 (35.3%)</td>
<td>7 (41.2%)</td>
<td>1 (5.9%)</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Relationship did not reach a statistical significance (\( p = 0.75 \)); (N – number of cases; +/–, 1+, 2+, 3+ – individual grades of Ki-67 positivity).

Fig. 1. Primary metatypical carcinoma – immunohistochemical staining for Ki-67 antigen. There is evident zonal staining up to 30% of all tumor cells (grade 2+), accentuated at the peripheral basaloid regions. Central squamous parts of cancer are virtually negative (clone MIB-1, DAKO, original magnification 240×; scale bar 125 µm).

Fig. 2. Primary nodular BCC with focal trichoepithelial growth features – immunohistochemical staining for Ki-67 antigen. There is irregular positivity approximately 35% of all neoplastic cells (grade 2+), while a certain part of tumor is strikingly positive, another regions are practically negative (clone MIB-1, DAKO, original magnification 240×; scale bar 125 µm).

adjacent to areas with a lower number of Ki-67 labeled cells (Figs 1–4). In general, superficial, infiltrative and morpheaform BCCs exhibited a diffuse pattern of immunoreactivity more frequently. Conversely, the most of nodular BCCs, BCCs with trichoepithelial features, and metatypical carcinomas displayed Ki-67 staining more commonly at the periphery of tumor formations. We subjectively failed to recognize any appreciable changes in the staining pattern between primary and recurrent tumors. Regardless of percentage range of Ki-67 staining, a nuclear immunoreactivity was intensive in almost all BCC cases. The vast majority of lesions with Ki-67 index exceeding 30% were accompanied by massive lymphocytic and plasmocytic infiltration around the neoplastic nests. Since these inflammatory cells also tended to show a nuclear Ki-67 positivity, this partially contributed to the difficulty in overall assessment of tumor growth fraction.

Discussion

In contrast to most other carcinomas, BCC of the skin is characterized by a relatively high percentage of proliferatively active tumor cells (Tilli et al. 2005). According to most studies published in the literature (Al-Sader et al. 1996; Matsuta et al. 1996; Horlock et al. 1997;
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Fig. 3. Recurrent nodular BCC – immunohistochemical staining for Ki-67 antigen. There is focal positivity approximately 5% of all neoplastic cells (grade +/−). Tumor recurred 7 months after treatment of primary lesion (clone MIB-1, DAKO, original magnification 240×; scale bar 125 µm).

Fig. 4. Recurrent nodular BCC with infiltrative growth features – immunohistochemical staining for Ki-67 antigen. There is diffuse positivity approximately 40% of all neoplastic cells (grade 2+). Tumor recurred 5 months after treatment of primary lesion (clone MIB-1, DAKO, original magnification 240×; scale bar 125 µm).

Till et al. 2002; Mazzarelli et al. 2003; Ionescu et al. 2006; Correa et al. 2009), mean BCC growth fraction detected immunohistochemically by antibodies against Ki-67 antigen ranged between 20–41%. In our study, the mean value of 27.4% is in agreement with the results of the above-mentioned papers. It is worth noting, however, some of the most serious and aggressive human malignances, such as multiforme glioblastoma, malignant melanoma or soft tissue sarcomas display a mean Ki-67 expression about 25% (Mahvash et al. 2011), 27% (Nasr & El-Zammar 2008) and 18% (Kazanowska et al. 2004), respectively. Thus, a proliferative activity of cutaneous BCCs in general reaches or even exceeds the prognostically most serious cancers. This observation raises the possibility that it may have partly different impact on evolution and biological behavior of this neoplasia. Moreover, the vast majority of the authors (Baum et al. 1993; Al-Sader et al. 1996; Horlock et al. 1997; Lee et al. 2006; Tilli et al. 2002; Mazzarelli et al. 2003; Ionescu et al. 2006) found that Ki-67 growth fraction varied considerably between different histomorphological BCC types, even in the tumors belonging to the same group. Our results have shown a wide percentage range in each histological BCC type as well, regardless whether they were primary or recurrent lesions. Although there probably does not exist a significant correlation between Ki-67 index and histopathological BCC types (Lee et al. 2000; Tilli et al. 2002), several papers indicate (Barrett et al. 1997; Horlock et al. 1997; Mazzarelli et al. 2003; Misiuk Hojo et al. 2005; Ionescu et al. 2006; Chuprov 2008) that aggressive tumor variants are generally accompanied by increased level of cell proliferation. In addition, it has been shown (Barrett et al. 1997) that the majority of infiltrative, sclerosing BCCs and metatypical carcinomas exhibited more extensive PCNA staining, in comparison to classic nodular BCCs. We also noted the highest mean value of tumor cell proliferation in infiltrative and morpheaform BCCs, but this relationship did not reach a statistical significance. To our knowledge, there is only one study (Chuprov 2009) declaring a significant association between increasing Ki-67 index and infiltrative BCC type.

It should be stressed, however, a major difficulty of objective immunohistochemical evaluation of BCC growth fraction is caused by the fact that this neoplasm usually displays very irregular and heterogeneous distribution of proliferatively active cells. Moreover, BCCs of the skin often manifest a combined morphology consisting of a mixture of several histopathological types, which may contain the cells with different growth potential. Additionally, it is of interest that areas with a high Ki-67 index often occur adjacent to the parts with only sporadical number of Ki-67 positive cells (Baum et al. 1993; Tilli et al. 2002; Cabral et al. 2004). Several authors have noted (Baum et al. 1993; Horlock et al. 1997; Tilli et al. 2002; Cabral et al. 2004) that in cutaneous BCCs, Ki-67-labeled cells may be confined either to the few rows of peripheral cancer cells, or they may be diffusely scattered throughout tumor nests. Cabral et al. (2004) distinguished two patterns of Ki-67 immunoreactivity in nodular BCCs. In small nodular pattern proliferation was limited to the basal palisading cells, whereas in large nodular pattern proliferation was absent at the basal membrane and distributed throughout the lesion. Many lesions contained both patterns in a side-by-side manner. They hypothesized that it perhaps reflects a loss of tumor differentiation and some mutations can result in the loss of cellular microarchitecture and proliferation control related to tumor-stroma interactions (Cabral et al. 2004). As a result, a cancer reverts to a non-regulated proliferation, diffusely distributed throughout the lesion. Coexistence of the various growth patterns in a single lesion supports a concept of bicolonality (or polyclonality) of neoplastic cells in cutaneous BCC. In our series, we were not able to objectively correlate Ki-67 staining patterns within the lesions regarding peripheral or diffuse, and thus, definitely segregate some histological subtypes. The main problem was that a majority of lesions had displayed a heterogeneous Ki-67 staining. For example, in certain areas, many tumors manifested typical peripheral staining, while in another parts, immunoreactivity ap-
peared to be much more diffuse and/or more irregular. There were only a few tumors with a “pure” peripheral and “pure” diffuse Ki-67 labeling. Therefore, we concluded that categorization of lesions into “diffuse staining group” and “peripheral staining group” would be very subjective, and thus, irrelevant for present study.

The fact that BCC of the skin shows relatively high percentage of proliferating cells deserves a special attention. It is not in line with the clinical finding that it is usually a slow-growing and indolent neoplasm with minimal metastatic potential (Tilli et al. 2005). This phenomenon could be explained either by a prolonged duration of the cell cycle, or a considerable and continual loss of cells accompanied by their permanent renewal (Baum et al. 1993). Already in 1972, Kerr & Searle (1972) supposed that a seemingly paradoxically slow growth rate of BCC might be due to a high apoptotic rate of tumor cells. This hypothesis was later supported by Mooney et al. (1995). At present, the differences in biological behavior and progression of cutaneous BCC are really explaining by imbalance between proliferative and apoptotic mechanisms of tumor cells (Tilli et al. 2002; Correa et al. 2009). Tilli et al. (2002) assumed that all BCCs composed of 3 cellular compartments, i.e. a progressive fraction protected from undergoing apoptosis, and proliferative and non-proliferative fraction, which are prone to apoptosis. Relative proportion of these compartments may differ in individual BCCs, and perhaps they could be changing depending on specific circumstances, which determine whether cells live or die.

Until now, conflicting data have been reported on the role of Ki-67 expression as prognostic factor. While some authors (Misiuk Hojlo et al. 2005; Chuprov 2008; Selim et al. 2009) claimed that an extent of Ki-67 expression was indicator of disease severity and useful prognostic parameter, the other investigators (Horlock et al. 1998; Cernea et al. 2005; Correa et al. 2009; Mateiu et al. 2011;) concluded that there were no statistically significant differences in BCC growth fraction between prognostically “favorable” and “unfavorable” lesions, and Ki-67 index could not be considered as a good prognostic marker. There are also controversial opinions about a relationship between BCC proliferative potential and tumor recurrences, which represent one of the most negative phenomena of this malignancy. Healy et al. (1995) and Yerebakan et al. (2003) found that Ki-67 expression was markedly higher in pBCCs which later recurred in comparison to non-recurrent tumors. Selim et al. (2009) observed that the percentage of Ki-67 labeling cells had shown significant increase in the relapsing tumors than that in the non-recurrent ones. On the other hand, Janisson-Dargaud et al. (2008) did not find significant differences in Ki-67 expression in pBCCs what had subsequent local recurrences in contrast to non-recurrent BCCs. Even our results are rather inclined to believe that tumor cells proliferation activity alone does not play a crucial role from this aspect. In addition, there is absolutely unexplained a relation between proliferative activity and metastatic potential of BCC, mainly due to the extremely rare cases of metastases. Ionescu et al. (2006) assessed 4 cases of metastasizing BCC and they found that metastases showed partly higher mean Ki-67 index in comparison with their corresponding primary lesions (62% vs. 51%). In one case, however, a percentage of Ki-67 expression was lower in metastatic tumor.

In conclusion, our present results indicate that a simple quantification of BCC proliferation activity alone is probably not sufficient for the prediction of further biological behavior, evolution and clinical outcome of this malignancy. Although infiltrative and morpheaform BCCs, known as aggressive tumor variants, displayed the highest Ki-67 index, immunohistochemical expression was too much variable in each histological tumor type to allow to be considered as reliable prognostic indicator. Anyway, in our opinion, due to some controversial views mentioned above, this relationship still remains to be better determined. We also think that further studies focused on the role of the Ki-67 protein in the cellular kinetics of cutaneous malignances and in the process of carcinogenesis of the skin should be useful.

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