

Expression of metallothionein I/II and Ki-67 antigen in various histological types of basal cell carcinoma

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Abstract: Basal cell carcinoma (BCC) is the most frequent skin cancer, with many different histological subtypes. Recent studies have investigated the expression of proliferative markers, but little is known about the expression of metallothioneins (MT) in different histological subtypes of this cancer and their impact on proliferation intensity in BCC. In this study, we examined MT-I/II expression by immunohistochemistry in 58 different histological subtypes of BCC (38 nodular, six adenoid, eight infiltrative, and six metatypic cases) and correlated its expression with tumor size and Ki-67 proliferation rate. Statistical analysis revealed no significant differences in the expression of studied markers in regard to the histological subtype. A positive correlation between MT and Ki-67 expression was observed for all the studied cases ($r = 0.26$; $p = 0.049$), but was even stronger in the metatypic subtype of BCC ($r = 0.85$; $p = 0.033$). MT and Ki-67 expression did not correlate with tumor size. In conclusion, it seems that metallothioneins may have an impact on the proliferation rate of BCC, but further studies are required to determine whether MT may be a risk factor of recurrences. (*Folia Histochemica et Cytobiologica* 2012, Vol. 50, No. 3, 352–357)

Key words: metallothionein, Ki-67, BCC

Introduction

Basal cell carcinoma (BCC, *carcinoma basocellulare*) represents a non-uniform group of tumors which are characterized by slow progression, and the very rare (below 0.1% of cases) development of metastases [1]. BCC is the most frequent invasive skin tumor in Europe, comprising around 80% of non-melanoma skin cancers [2]. In most cases, it involves slowly growing tumors of a relatively low aggressiveness. A small minority of BCC leads to relapses and local infiltration of tissue structures which may lead to death of the host [2].

Depending on histological and clinical traits, more than ten forms of the tumor can be distinguished [3]. The isolation of individual types of BCC is useful due to the variable character of its development (variable aggressiveness), which determines the selection of an appropriate therapeutic approach [4, 5]. The nodular and superficial forms manifest no particular aggressiveness and can often be cured. The varieties manifesting higher aggressiveness and more frequent recurrences include micronodular, morpheaform, infiltrative and metatypic types. They may aggressively infiltrate and destroy surrounding tissues, and in specific conditions may endanger the patient's life [6]. The superficial type manifests no particular aggressiveness but frequently shows recurrences [5]. Due to the frequent coexistence of various forms of BCC within a single lesion and, occasionally, unpredictable behavior of the tumor, the identification of markers which are independent of histopathological structure

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Table 1. Patient and tumor characteristics

	No. of cases (%)	Age (years) Mean (range)	Tumour diameter [mm] Mean (range)
BCC	58 (100)	65.98 (21–87)	20.63 (8–70)
Adenoid BCC	6 (10.3)	62.17 (52–72)	21.33 (15–30)
Infiltrative BCC	8 (13.9)	64.13 (47–81)	21.71 (12–35)*
Metatypic BCC	6 (10.3)	71.67 (45–85)	30.00 (10–70)
Nodular BCC	38 (65.5)	66.08 (21–87)	18.84 (8–50)

*Data missing for one case

but would be helpful in establishing a prognosis, is urgently needed [2, 5].

Expression of metallothionein (MT) in tumor cells represents a relatively new prognostic index in various neoplastic diseases, as indicated by numerous studies. MT is a low molecular weight protein (around 7 kDa) which is widely expressed in cells (nuclei and/or cytoplasm) of various organs and tumors [6, 7]. Depending on the type, MT consists of 61–68 amino acids, forming two chains of α and β , linked by a lysine dimer. Considering their structure and manifestation, four basic types of MT can be distinguished, including MT-I, MT-II, MT-III, MT-IV [6, 7]. Binding of heavy metal ions, either indispensable for MT function or toxic, represents a principal character of MT. The protein plays a protective role, preventing against intoxication with heavy metals, such as Pb, Hg, Cu, Cd [8, 9]. MT manifests also a strong anti-oxidative activity, protecting cells from the damaging effects of reactive oxygen species, ionizing radiation and chemotherapeutic agents [10–12]. Intensely dividing cells (including tumor cells) manifest an increased expression of MT, which supplies zinc (Zn) ions for enzymes involved in DNA replication and protects cells from apoptosis [13]. This has induced investigators to examine the expression of MT in various tumors [14–16]. An increase in MT expression in tumor cells correlates with expression of Ki-67 proliferation antigen and a less favorable outcome of the disease [6, 7, 14–16]. It has been shown that MT-2A isoform expression in breast cancer cells stimulates cell proliferation, but does not exert anti-apoptotic properties [17]. Moreover, a decline of MT-I/II expression is associated with a decrease of PCNA and Ki-67 expression both in sun-exposed and sun-protected skin, which may indicate its impact on cell proliferation [18]. It has been suggested that MT-I/II may protect skin tissues from ultraviolet radiation (UVR) induced carcinogenesis on the one hand, but may induce tumor growth once these occur [19].

Until now, only individual studies have been published in which MT expression was studied in skin

cancers [20, 21]. The studies showed an increased expression of MT in spinocellular carcinoma and a less typical increased expression of MT in aggressive varieties of BCC, but none of the investigations dealt with the relationship between expressions of MT and Ki-67 antigen in various histological types of BCC.

Our study aimed at determining the intensity of MT-I/II expressions using the immunohistochemical method in various histological types of BCC, and correlating its expressions with the expression intensity of Ki-67 antigen and tumor size.

Material and methods

Patients. Studies were performed on the material of various histological types of basal cell carcinomas (58 cases) obtained during excision of the tumor in the Department of Dermatology, Venerology and Allergology, Medical University in Wrocław between 2005 and 2007. The clinical and pathological data are summarized in Table 1. The study was approved by the Commission of Bioethics at Wrocław Medical University.

Immunohistochemistry. The tumor samples were fixed in 10% buffered formalin, dehydrated and embedded in paraffin blocks, of which hematoxylin and eosin (HE) stained slides were made and used for verification of the histopathological diagnosis. All immunohistochemical reactions (IHC) were performed on 4- μ m-thick paraffin sections mounted on Superfrost Plus slides (Menzel Gläser, Braunschweig, Germany). First, sections were deparaffinized in xylene and rehydrated. In the case of anti-Ki-67 IHC reactions, the sections were incubated in a citrate buffer (pH 6, 10 mM; 95°C, 20 min) in order to retrieve the antigens. Activity of endogenous peroxidase was blocked by 5 min incubation with 3% solution of H₂O₂. Expression of MT-I/II and Ki-67 antigen was demonstrated using mouse monoclonal anti-MT-I/II (clone E9, dilution 1:100) and anti-Ki-67 (clone MIB-1, dilution 1:100) antibodies, respectively. Sections were incubated with primary antibodies overnight at 4°C. The studied antigens were visualized using biotinylated antibodies and streptavidin, conjugated with horseradish peroxidase

(LSAB+ System-HRP). Diaminobenzidine (DAB) served as a substrate. The reactions were accompanied by a negative control. Subsequently, the preparations were counterstained with hematoxylin. All the antibodies and reagents originated from DakoCytomation (Glostrup, Denmark).

Evaluation of IHC reactions. The intensity of MT expression in tumor cells was evaluated using the semi-quantitative technique of Remmele and Stegner [22]. In every case, the semi-quantitative immunoreactive score (IRS) of IHC was calculated by multiplying the grade of scale defining proportion of positive cells (0–4) by the degree of scale defining intensity of color reaction (1–3), yielding a final score of 0 to 12. Intensity of Ki-67 antigen expression in tumor cells was evaluated by the proportion of positive cells among tumor cells as follows: 0 pts — no reaction; 1 pt — 1–10%; 2 pts — 11–25%; 3 pts — 26–50%; 4 pts — > 50% positive cells.

Statistical analysis. The obtained results were subjected to statistical analysis using Prism 5.0 software (GraphPad, CA, USA). Shapiro–Wilk test was applied to estimate the normality of distribution. Spearman’s correlation test, the Kruskal–Wallis test, and the U test of Mann and Whitney were used to compare the results. Differences were accepted to be significant at $p < 0.05$.

Results

Cytoplasmic/nuclear expression of MT and nuclear expression of Ki-67 antigen were detected in all examined cases of the studied basal cell carcinomas (Figure 1). The highest intensity of MT-I/II expression was noted in adenoid BCC (5.17 ± 2.93), and the lowest in metatypic BCC (4.33 ± 1.97). The most pronounced expression of Ki-67 antigen was detected in the metatypic type of BCC (2.66 ± 1.03), and the lowest intense one in nodular BCC (1.90 ± 0.89). Expression intensities of the studied antigens for all the examined cases and individual histopathological types are presented in Table 2. No significant differences were detected in MT-I/II expression or Ki-67 expression between individual histological types of BCC.

A positive correlation was demonstrated between expression intensities of Ki-67 antigen and MT-I/II, when the analysis took into account all 58 studied cases of BCC ($r = 0.26$; $p = 0.049$; Figure 2A). Among the studied subtypes, only the metatypic BCC subtype demonstrated a strong positive correlation between the two studied markers (MT-I/II and Ki-67) ($r = 0.85$; $p = 0.033$; Figure 2B). In cases of the other studied subtypes, no significant correlation was observed between the studied markers.

Neither in the entire studied group, nor within individual histopathological types, could any relation-

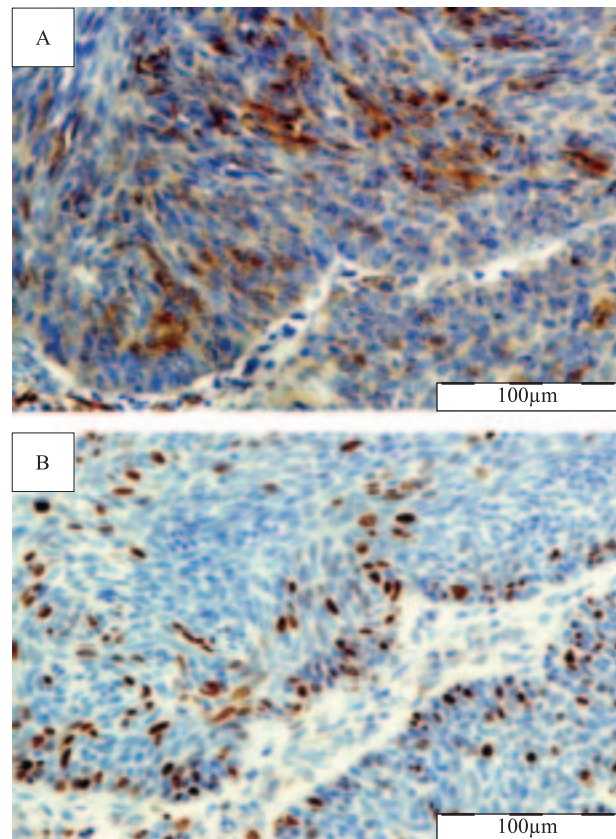


Figure 1. Cytoplasmic/nuclear expression of MT-I/II (A) and nuclear expression of Ki-67 antigen (B) in cells of BCC

Table 2. Expression of Ki-67 antigen and MT I/II in histological subtypes of BCC

	Ki-67 expression (Mean \pm SD)	MT-I/II expression (Mean \pm SD)
BCC	2.02 \pm 0.93	4.50 \pm 2.44
Adenoid BCC	2.17 \pm 0.98	5.17 \pm 2.93
Infiltrative BCC	2.00 \pm 0.93	4.63 \pm 1.60
Metatypic BCC	2.67 \pm 1.03	4.33 \pm 1.97
Nodular BCC	1.90 \pm 0.89	4.40 \pm 2.64

ship be detected between intensity of Ki-67 antigen or MT-I/II expression on the one hand and diameter of the tumor on the other.

Discussion

Since the mid-1990s, numerous studies have been published related to expression of various antigens, using e.g. IHC in skin carcinomas (mainly basal cell carcinomas and spinocellular carcinomas). In these studies, the authors have suggested that a variable expression of certain antigens in tumor cells (in the cell nucleus or in the cytoplasm) or in cells of their sublay-

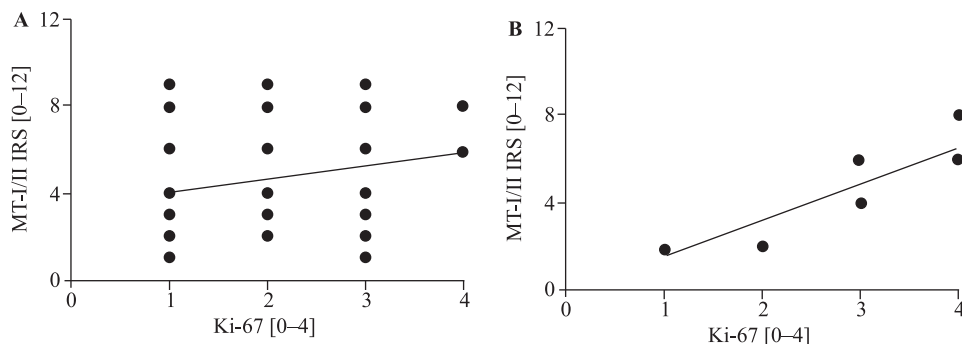


Figure 2. Correlation between expression of MT-I/II and Ki-67 antigen in all the analyzed samples (A) and metatypic BCC (B)

er may correlate with the aggressiveness of the tumors, providing important prognostic indices. Potential advantages have also been suggested of using IHC studies for easier differentiation between individual varieties of BCC as well as between BCC and tumors of a similar histomorphology (e.g. trichoepithelioma) [21, 23]. Results obtained in IHC studies conducted on these types of tumors have frequently been equivocal.

Ionesco et al. performed a comparative analysis of Ki-67, p53 and Bcl-2 antigen expression in two groups: the more aggressive (metastatic) BCC tumors and BCC of a benign course. They demonstrated no significant differences between the groups in expression of the antigens [24]. On the other hand, Yerbakan et al. examined expression of Ki-67, CD31 and epidermal growth factor receptor (EGFR) in groups of BCC of variable aggressiveness. They found that expression intensity of the mentioned antigens was significantly higher in recurrent (i.e. more aggressive) tumors, compared to primary tumors [25]. In a similar way, elevation in Ki-67 expression level was detected by Healy et al. in BCC tumors which relapsed after treatment, compared to tumors which were cured [26]. The cellular proliferation characteristics of various histological/clinical types of BCC were analyzed by Horlock et al. using analysis of Ki-67 antigen expression [27]. Their investigations showed that intensity of proliferation measured by the level of Ki-67 antigen expression was lower in the nodular and micronodular types, and higher in scleroderma-like, infiltrating and superficial BCC types, which is consistent with our observations. According to the authors, a correlation can be noted between higher expression of Ki-67 antigen and BCC variations of higher aggressiveness [27]. However, the authors seemed to pay no attention to the significant aggressiveness manifested by the micronodular type of BCC, manifesting in their hands low levels of Ki-67 antigen expression [4, 27]. Other proliferation markers, including PCNA and Ki-67 in various types of BCC, were also analyzed by Barrett et al. [28]. In this study, nu-

clear reaction of PCNA expression demonstrated higher intensity than expression of Ki-67. PCNA was manifested in less than 10% of BCC cells in the nodular form, in more than 30% of cells in most tumors in the scleroderma-like and infiltrating forms, and in all tumors of the metatypic form [28]. Another study which compared intensity of Ki-67 antigen expression to depth of infiltration and histological type of BCC demonstrated no relationship between the parameters [29].

The results of the above-mentioned studies are consistent with the results obtained by us showing no differences between histological types of BCC and tumor size. Janisson-Dargaud et al. also demonstrated no differences in the expression of proliferation markers between recurrent and non-recurrent forms, and only the frequency of aneuploidy proved to represent a prognostic factor linked to the risk of recurrence [30].

Rossen et al. analyzed levels of expression of MT-I/II isoforms in BCC compared to normal epidermis and benign epidermal hypertrophy, including basaloidal hypertrophy, covering solid fibromas [20]. Within normal epidermis, the expression of MT-I/II was documented in cytoplasm of basal cells. A similar form of MT expression was demonstrated in a hypertrophic epidermis. Basaloidal proliferations, as well as superficial and nodular forms of BCC, demonstrated a decreased MT-I/II expression (in approximately 92% of the cases) [20]. Nevertheless, in 86% of cases of infiltrating BCC, increased expression of MT-I/II was demonstrated in neoplastic cells [20]. Our results seem to confirm this observation. The increased expression of MT-I/II in infiltrating BCC type may be linked to an increase in clinical malignancy. Interestingly, the metatypic BCC showed comparable low MT-I/II expression with the nodular BCC subtype, although the clinical course of the latter is more benign. Moreover, in the metatypic BCC, a weak correlation between MT-I/II and Ki-67 antigen expression was noted, which may

point to more pronounced pro-proliferative effects of MT-I/II expression in this particular type. Although metatypic BCC is regarded as an aggressive subtype of BCC, it manifested the lowest MT-I/II expression of the analyzed subtypes. We used IHC techniques to determine MT-I/II expression, but recent studies have shown that only particular MT-I/II isoforms may contribute to cancer cell aggressiveness in some tumor types [31–35]. In our study, we noted the highest Ki-67 antigen expression in this subtype. Other investigations related to the expression of MT in BCC, SCC and healthy skin have also demonstrated an increased level of MT-I/II expression in neoplastic tissues, which may point to the role of MT-I/II in the progression of basal cell carcinoma [21].

Even if our studies have failed to document significant differences in the expression of Ki-67 antigen and MT-I/II expression between various histological types of BCC, its nodular type, manifesting the most benign clinical course, has shown the lowest level of Ki-67 antigen expression and a comparable expression of MT-I/II compared to the other histological types. The positive correlation between expression of Ki-67 and MT-I/II is worth accentuating, as it confirms the role of MT-I/II in the proliferation of neoplastic cells [6, 7, 13]. Moreover, in the metatypic variety of BCC, the correlation coefficient has proven to be very high despite the low number of examined cases, which may indicate a significant effect of MT-I/II on the proliferation process in this histological type of BCC. In this study, MT-I/II and Ki-67 antigen expression did not correlate with tumor size, and the absence of unequivocal data related to the risk of recurrence does not permit the recognition of Ki-67 antigen or MT-I/II as prognostic factors in BCC, but does give new insights into the biology of BCC. However, our study was limited by the small number of particular cases of BCC subtypes. Therefore, our findings cannot be generalized. Further studies are necessary to confirm the role of MT-I/II in different BCC subtypes.

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