The Expression of MT1 Melatonin Receptor and Ki-67 Antigen in Melanoma Malignum

KAROLINA DANIELCZYK1 and PIOTR DZIEGIEL 1,2

1Department of Histology and Embryology, Wrocław Medical University, 50-368 Wrocław; 2Department of Histology and Embryology, Poznań University of Medical Sciences, 61-781 Poznań, Poland

Abstract. Background: Melatonin, the principal hormone produced by the pineal gland, manifests strong potency of inhibiting growth of dermal melanoma cells both under in vitro and in vivo conditions. Although the mechanism of the phenomenon has not been fully clarified yet, melatonin receptors seem to play a key role in the inhibition. In humans, two main types of high affinity membrane melatonin receptors have been identified, including MT1 (Mel1a) and MT2 (Mel1b) receptors, and their expression increases efficacy of the oncostatic melatonin activity. The principal aim of this study involved determination of location and intensity of expression of MT1 melatonin receptors and of Ki-67 proliferation-associated antigen in dermal melanoma using an immunohistochemical technique and an examination of their reciprocal correlation and their relationship with clinical advancement of the tumour, i.e., with its depth of infiltration. Patients and Methods: Immunohistochemical studies were conducted on the material of 48 cases of dermal melanoma, including 38 primary tumours and 10 metastatic lymph nodes, fixed in formalin and embedded in paraffin. Results: In all the examined cases, positive immunohistochemical reactions were obtained with antibodies to MT1 and Ki-67. Expression of MT1 receptor was more pronounced in primary tumours than in lymph nodes (p<0.05). Depth of tumour infiltration demonstrated a moderate positive correlation with the intensity of MT1 expression (r=0.45; p<0.05) and a strongly positive correlation with the expression of Ki-67 antigen (r=0.79; p<0.05). Moreover, both in primary tumours and in metastatic lymph nodes, a weak correlation was detected between the expression of MT1 receptor and expression of Ki-67 antigen. Conclusion: Confirmation of positive correlation between the expression of MT1 receptor and depth of melanoma infiltration may point to future use of MT1 as a prognostic index for such tumours.

In the past 20-30 years, much attention has been focused on the biologically active substance generally known as melatonin. Melatonin is broadly represented in both the animal and plant kingdoms. It was identified even in bacteria, unicellular eukaryotes, macroalgae, in numerous species of avertebrates and vertebrates (1). In vertebrates melatonin is synthesized mainly in pinealocytes of the pineal gland, the endocrine gland of epithalamus (1, 2). Apart from the pineal gland, melatonin is also synthesized in, e.g., retina, Harder’s lacrimal glands, alimentary tract, human and mouse bone marrow cells, blood platelets and lymphocytes (1). Secretion of melatonin is synchronized with the light/dark cycle and provides information to the body on the duration of day and night (1). Its secretion increases soon after sunset, peaks between 2 and 4 a.m. and gradually decreases during the second half of the night. Peak levels of the substance in blood are specific to individuals and they change with progressing age. In young individuals, the blood melatonin level is in the range of 54-75 pg/ml, while in older individuals it decreases to 18-40 pg/ml (2, 3). In blood, 70% of melatonin circulates in the form of complexes with albumins and glycoproteins (3). Since the hormone is a small, lipophilic molecule, it can also diffuse into other fluids (saliva, cerebro-spinal fluid, lymph, seminal fluid, fluid of ovarian follicles, aqueous humor in the anterior chamber of the eye) (3, 4). Melatonin is metabolized in the liver and partially in kidneys. It undergoes hydroxylation to 6-hydroxymelatonin by cytochrome P450 monooxygenases. The arising 6-hydroxymelatonin sulphate or glucuronate is excreted with urine (1, 4).

In mammals, current reports most frequently present four principal mechanisms of melatonin activity. Among these, binding to intracellular proteins, such as calmodulin (5), anti-oxidative function (5-9), binding to nuclear receptors (10) and binding to melatonin receptors located in the cell...
membrane (11-12) are particularly important. The latter is the most significant for our study of the mechanism of melatonin activity. Advanced studies with the use of molecular biology techniques permitted to distinguish two main subclasses of receptors to be distinguished namely ML1 (13) and ML2 (14) receptors. ML1 membrane receptors belong to G protein-coupled receptors (GPCR) superfamily. In addition, among receptors of this group, three subtypes were distinguished, including MT1 (Mel1a), MT2 (Mel1b) and Mel1c. They manifest high affinity and bind melatonin at picomolar concentrations of the hormone (15). Among the melatonin membrane receptor types, only MT1 and MT2 receptors were identified in mammals (13). The subtypes manifest 60% homology at the level of amino acid sequences (16). Development of autoradiographic detection techniques permitted a more accurate morphological localization of melatonin receptors in various tissues types in the central nervous system, circulatory system, genital system and alimentary system (17, 18).

MT1 receptors are coupled to the pertussis toxin-sensitive G proteins. With mediation of protein G α subunit, MT1 receptors inhibit activity of an effector protein, namely adenyl cyclase and in this way they reduce the amount of cAMP produced (11, 19).

A change in cAMP intracellular concentration allows, among others, for a control of activity of protein kinase C (PKC), protein kinase A (PKA), mitogen – activated protein kinase (MAPK), potassium channels, of cGMP level, or of intracellular levels of Ca²⁺ (5). The lower activity of PKA in the signalling pathway exerts a negative effect on the phosphorylation level of transcription factors, i.e. cAMP response element-binding (CREB) and expression of specific genes (20-21). In an indirect way, PKA is also engaged in control of Raf protein activity, which participates in processes of proliferation, angiogenesis, cell differentiation and migration (22). The effect of melatonin on cell division has been confirmed in studies conducted, among others, on S-19 and B-16 cell lines of mouse melanoma cells, cells of iridial melanoma and on cells of breast cancer (4, 23-25).

Ki-67 antigen represents a non-histone nuclear protein with a molecular weight of 345-395 kD. Ki-67 is present in cells throughout active phases of the cell cycle (G 1, S, G 2 and mitosis) (26-27). It is absent in cells at the resting G 0 phase of the cell cycle (26, 28-29).

Ki-67 index defines the tumour growth fraction and provides a recognized prognostic index of survival in patients with lymphoma, breast cancer, colon cancer, melanoma and other tumours (26, 29-30).

Melanoma is a tumour arising from neoplastically transformed melanocytes. It develops most frequently in the skin but it may appear anywhere where melanocytes, which contain and produce melanin, are present, e.g. on mucous membranes (in oral cavity, rectum) or inside the eye-bulb. Its exceptional malignancy reflects its extremely high tendency to develop metastases and the relatively low curability (31).

At present, diagnosis and prognosis in dermal melanoma represent the resultant of clinical evaluation (characteristics of the naevus, number of involved lymph nodes) and histopathological appraisal (depth of infiltration, ulceration) (32). Unfortunately, melanoma manifests such a high ability to develop metastases that they appear even in patients with tumours of an insignificant clinical stage. In early stages of the disease, it is extremely difficult to properly define prognosis, including the risk for spread, mainly through lymphatic and less frequently via blood vessels, using a routine diagnostic approach. Additional laboratory tests, including immunohistochemical tests may supply data useful for diagnosis of the tumour, for defining its stage, monitoring of therapeutic efficacy, in prognosis of a relapse of the disease and, thus, for its prognostic and predictive evaluation (33).

In the case of melatonin receptors, representing the main topic of this study, a potential relationship exists with oncostatic properties of melatonin as well as expression of MT1 receptors correlating with progression of melanoma. Using immunohistochemical techniques to evaluate MT1 receptor expression we tested the hypothesis. Present study aimed at evaluation of MT1 receptor expression, as related to Ki-67 antigen expression and clinical and pathological data, in dermal melanomas.

**Patients and Methods**

**Samples.** The studies were performed taking advantage of paraffin-embedded tumour sections sampled from patients with diagnosis of dermal melanoma. Forty-eight archival paraffin blocks were obtained from the Lower Silesian Centre of Oncology in Wroclaw sampled in the years of 2000-2004. The paraffin blocks were cut on Leica RM 2145 microtome to sections of appropriate thickness of 7 µm (classical histological examination) or 4 µm (immunohistochemical tests). The so prepared biological material was used to conduct histological studies (staining with hematoxylin and eosin) and immunohistochemical studies with use of antibodies to melatonin receptors MT1 and Ki-67 antigen.

**Histological and immunohistochemical reactions.** Classical histological examination with hematoxylin and eosin staining was performed in a routine manner while reactions for the immunohistochemical reactions were individually ascertained for presence of MT1 receptor and Ki-67 antigen.

The sections were deparaffinized in xylene and, then, they were rehydrated by passing them through a series of alcohol of a decreasing concentration. The epitopes were unmasked by incubating preparations in a water bath in a citrate buffer (pH 6, 10 mM) at 96-98˚C for 7 min. Endogenous peroxidase was blocked by incubating the sections in 3% hydrogen peroxide while non-specific binding sites were blocked using Antibody Diluent (30 min).

For detection of MT1 receptors the sections were incubated with the primary antibody to the second cytoplasmic domain of MT1...
receptor, (polyclonal rabbit antibody # A99205, diluted at 1:600; Millipore, Canada) for 1 h at room temperature. Goat secondary antibodies (EnVision™/HRP, Dako, Denmark) directed to rabbit immunoglobulins, were bound to a dextran framework, conjugated with peroxidase. The reaction was developed using 3,3'-diaminobenzidine tetrahydrochloride (DAB). Product of the reaction manifests an intensely brown colour and is located at the site of antigen presence. Detection of Ki-67 was conducted using mouse monoclonal primary antibody (clone MIB-1), diluted at 1:50. The incubation took 18 h at the temperature of 4°C. Subsequently, biotinylated secondary antibodies and peroxidase-labelled streptavidin (Universal DakoCytomation LSAB®+, Dako) were applied. The sections were consecutively incubated with a solution of DAB. The reaction resulted in a deposit of a brown insoluble and stable product at the site of the antigen.

Final stages of the immunohistochemical reaction, according to the standard procedure, involved counterstaining with hematoxylin and passage through a series of alcohol of an increasing concentration. Finally the preparations were sealed in Euparal (Roth, Germany).

**Evaluation expression of MT1 receptor and of Ki-67 antigen.** The histological preparations were divided into two groups: the group of primary tumours and the group of metastatic lymph nodes.

Results of H+E staining (only the cases of primary tumours) were evaluated according to the four stage scale of Breslow. Thickness of the tumour (depth of infiltration), providing the basis for the classification, from epidermal granular layer to the level of the deepest penetration (34) was measured under a microscope. In the studied tumours, the depth of infiltration was measured in mm using an Olympus BX-41 light microscope (Japan), coupled to the visual pathway, and software for computer-assisted image analysis, Analysis 3.2 (Olympus, Germany).

Expression of MT1 receptor was evaluated using an Olympus BX-41 light microscope and the semiquantitative immunoreactive score (IRS) technique of Remmele and Stegner (35). The scale takes into account both the content (%) of cells with a visible reaction product (A) and intensity of the reaction (B). The final result, ranging from 0 to 12 points, is the product of the scores (A x B) (Table I).

Intensity of Ki-67 antigen expression was evaluated under an Olympus BX-41 light microscope. In every preparation, three fields were delineated with the most intense expression of Ki-67 antigen expression, enclosing approximately 100 cells, among which the number of positive cells was scored (Hot Spot technique). The final result was the mean score obtained in the three fields. Expression of Ki-67 was also appraised using the IRS according to the criteria of Remmele and Stegner (35) (Table I).

**Statistical analysis.** The obtained results were subjected to statistical analysis using Statistica version 7.1 software (StatSoft; Poland). When groups of data were compared which failed to exhaust assumptions of parametric tests, e.g. the Mann-Whitney U-test, the non-parametric equivalent of Student’s t-test was employed. Correlations were analyzed calculating Spearman’s rank correlation coefficient. In all the analyses, results were assumed to be significant at p<0.05.

**Results**

The studies were conducted on 48 cases with diagnosis of dermal melanoma, among which 38 cases were of primary tumours and 10 represented metastatic lymph nodes.

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Table I. Semi-quantitative IRS scale according to Remmelle and Stegner (35).

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<tr>
<th>A</th>
<th>B</th>
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<tr>
<td>0 No positive cells</td>
<td>0 No colour reaction</td>
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<tr>
<td>1 ≤10% positive cells</td>
<td>1 Low colour intensity</td>
</tr>
<tr>
<td>2 11% -50% positive cells</td>
<td>2 Moderate colour intensity</td>
</tr>
<tr>
<td>3 51%-80% positive cells</td>
<td>3 Intense colour reaction</td>
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<td>4 80% positive cells</td>
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**Depth of infiltration.** Depth of neoplastic infiltration was assessed in 38 cases of primary tumours. According to the scale of Breslow, more than half of the examined cases demonstrated III (39%) or IV (26%) degree of infiltrate depth, which pointed to the high malignancy of the tumours and a most unfavourable prognosis. The criteria revealed II degree of infiltration in 24% cases and I degree of infiltration in the remaining 11% of the cases.

**Expression of MT1 receptor and of Ki-67 antigen.** Immunohistochemical reaction detecting MT1 receptors presented intensely brown product located at the site of MT1 receptors. To a significant extent, the reaction manifested a membraneous-cytoplasmic location since the employed antibodies were targeted at the second cytoplasmic domain of the MT1 membrane receptor (Figure 1).

The immunohistochemical reaction detecting MT1 receptor protein was performed for 38 cases of primary tumours and 10 metastatic lymph nodes. In all the cases, a positive reaction was observed. Using the IRS scale, individual primary tumours manifested an average MT1 expression of 6.71 (SD±3.19). This pointed to an abundant expression of MT1 receptors. To a significant extent, the reaction manifested a membraneous-cytoplasmic location since the employed antibodies were targeted at the second cytoplasmic domain of the MT1 membrane receptor (Figure 1).

In over half of the cases (in 24/38), fewer than 5% of the cells demonstrated the presence of the antigen. In five cases the expression was demonstrated in ~20% cells, while in the remaining cases the expression was noted in ~10% nuclei of melanoma cells. On average, 7.8% of cells of the examined melanomas manifested expression of Ki-67 antigen. In the examined lymph nodes, the expression was almost twofold higher, being on average 15% of melanoma cells. However, the difference in Ki-67 antigen expression between primary tumours and metastatic lymph nodes proved to be insignificant.
Correlation between MT1 receptor expression, expression of Ki-67 antigen and depth of tumour infiltration. The obtained data were subjected to statistical analysis and significance of correlation was examined between expressions of MT1 receptor, Ki-67 antigen and the degree of tumour infiltration depth. In line with the criteria of Remmele and Stegener, individual preparations manifesting expression of MT1 receptor and expression of Ki-67 antigen were given scores ranging from 0 to 12 points. In order to compare expression levels of the two proteins, the results were grouped according to the score: (+++) 6-12 pts. strong expression; (+++) 3-4 pts., average expression; (+) 1-2 pts., weak expression, and the percentages of the cases for individual groups were calculated.

More than 70% of the examined primary tumours demonstrated high expression of MT1 receptor, within the range of 6-12 points (+++). The opposite phenomenon was noted for Ki-67 antigen: close to 70% of the cases manifested scores of 1-2 points (+). In lymph nodes, similarly to primary tumours, high expression of MT1 was observed in 50% of the cases and was accompanied by a weak expression of Ki-67 antigen. The results suggested that high expression of melatonin MT1 expression was accompanied by low Ki-67 proliferation index. The relationship between MT1 and Ki-67 expressions was examined using Spearman’s correlation analysis (Figures 4 and 5). In both examined groups of cases, a weak or a very weak correlation was disclosed between MT1 and Ki-67 antigen expression, both in the primary tumours (r=0.19; p<0.05) and in the lymph nodes (r=0.12; p<0.05).

An attempt was also made to demonstrate relationship between the level of MT1 receptor expression and the degree of tumour infiltration depth. The number of cases manifesting the highest level of MT1 expression (+++), was found to increase with increasing tumour depth of infiltration. Tumours in the (++++) group were frequently (12/38) characterized by a tumour infiltration depth of 1.5-4 mm (III degree). Spearman’s correlation analysis documented a significant, moderate positive correlation between the depth of tumour infiltration and the expression level of melatonin MT1 receptor (r=0.45; p<0.05); (Figure 6).

Similarly, using the correlation test, a strong positive correlation was found between expression of Ki-67 antigen (evaluated by percentage of positive cell nuclei) and the depth of tumour infiltration (r=0.8; p<0.05); (Figure 7).

Discussion
Results of studies conducted to date, performed on numerous experimental models, point to the oncostatic effect of melatonin (36). Both physiological and pharmacological concentrations of the hormone inhibit tumours of various type under in vitro and in vivo conditions (37-44). Melatonin used in adjuvant therapy enhances efficacy of chemotherapy and alleviates negative effects of using the drugs (7). It may also inhibit development and progression of various tumours (3, 7, 25).

Among other data, a significantly higher incidence of dimethylbenzanthracene (DMBA)-induced tumours has been disclosed in pinealectomised than in non-operated control rats (45). In addition, administration of melatonin effectively reduces the risk of developing a tumour (45). Numerous studies are being conducted on cell cultures, aimed at verification of the anti-proliferative effect of melatonin on tumour cells (3, 2). Inhibitory effect of the hormone has been verified mainly in cases of MCF-7 breast cancer cells, HT-29 colon cancer cells, M-6 human melanoma cells, HEPA1-6 mouse liver cells, SK-N-MC neuroblastoma cells, PC12 chromaffin cells of phaeochromocytoma and LNCaP prostate cancer cells, (37-44, 46).

Melatonin, as a small molecule of an amphilous nature, easily penetrates several compartments of a cell. It may act directly, independently of receptors or with mediation of receptors, which significantly impedes interpretation of its activity in individual cases. An effect of the hormone on tumour development may reflect its anti-oxidative properties and its inhibitory effect on calmodulin MEK/ERK signalling cascade with the use of melatonin receptors, MT1 and MT2 (5-8, 47-48).

Earlier studies on melatonin receptors were based mainly on binding of appropriate radioligands, in vitro autoradiography, biochemical techniques and experiments conducted on cell cultures and tissues such as retina, skin, mammary gland, colon and brain (49). The first reports on MT1-specific antibodies appeared in the paper of Brydon et
al. (50), but until 2002, no data were available on any attempts to use the antibodies in immunohistochemical analysis of tissues other than the brain. It was the research group of Dillon et al. (49) who for the first time examined expression of MT1 receptors in healthy mammary gland tissue and in paraffin-embedded sections of tumours of the organ. The authors analyzed the relationships between MT1 expression on one hand and selected clinico-pathological prognostic parameters on the other (49). Their results failed to verify correlations between MT1 expression on one hand and tumour size, patient’s age, number of removed and positive lymph nodes, estrogen and progesterone receptors, or HER2 receptors on the other. Nevertheless, they indicated a positive correlation between expression of MT1 receptors and degree of malignancy in tumours of mammary gland (49). Results of recent immunohistochemical studies on MT1 receptors provide further proof for higher levels of the receptors in neoplastically transformed than in normal mammary gland tissue (51). In addition, in contrast to results of Dillon et al. (49), an inverse correlation was observed between expression of MT1 receptors and levels of expression of estrogen (ERα) and progesterone (PR) receptors, suggesting that an augmented expression of MT1 receptors is accompanied by low levels of ERα and PR (51).

Taking into account that melatonin, acting through MT1 receptors, lowers transcriptional activity and expression of ER and PR receptors and may assist in lowering the probability for remission of receptor-positive breast cancer following hormonal therapy (51).

The main research aim of this study involved determination of expression and localization of melatonin receptors, MT1, in melanomas using an immunohistochemical technique. Melatonin has been found to inhibit growth of selected cell lines of melanoma, originating from human or rodent skin, choroid membrane of the eye (23, 33, 52). The research group of Slominski has examined the inhibitory effect of melatonin on four melanoma cell lines, representing distinct stages of growth and phenotype (53). In its pharmacological concentrations of $10^{-3}$-$10^{-7}$ M, melatonin inhibited cell proliferation in every studied line, beginning at cells in the

![Figure 1. Immunohistochemical reaction for MT1 receptor protein. Malignant melanoma. Tumour cells with membranous-cytoplasmic immunohistochemical reaction are evident, pointing to the presence of MT1 receptors. Magnification: x400.](image1)

![Figure 2. Expression of MT1 melatonin receptors in primary tumours and in lymph nodes. The difference was significant at p<0.05.](chart1)

![Figure 3. Immunohistochemical reaction for Ki-67 antigen in malignant melanoma. Melanoma cell nuclei with brownish reaction product, pointing to expression of Ki-67 antigen. Magnification: x200.](image2)
radial growth phase (SBCE2), through cells in the vertical growth phase (WM-98), metastatic cells (WM-164), up to melanin-producing cells (SKMEL-188) (45). Similar results have been obtained for B16 mouse melanoma cells and M6 human melanoma cells (46, 53).

Expression of membranous melatonin receptors may significantly influence effects of melatonin (49). Studies on tumour tissues demonstrate differences in intensity of their expression (49). For example, low level of MT1 expression has been noted in human epithelial cells of gallbladder adenocarcinoma while MCF-7 breast carcinoma cells, manifest higher than normal level of MT1 receptors (49, 54). Presence of MT1 receptors in MCF-7 breast carcinoma cells and in S-91 mouse melanoma cells augments oncostatic activity of the hormone, which may be of significance for the possible application of melatonin in therapy (24, 43, 49). In the case of melanoma cells of ocular choroid membrane, melatonin inhibits growth exclusively in tumour cells, sparing growth of normal melanocytes (52). The observations suggest a variable expression of the membrane receptors, dependent on the type of cell involved, which may be used in potential evaluation of melanoma cells sensitivity to melatonin treatment.

MT1 receptors represent the principal melatonin-binding site in human skin (55). Their presence has been demonstrated on keratinocytes, melanocytes, fibroblasts, in melanoma cells and in cells of squamoepithelial cancer (16). Earlier reports related to distribution of MT1 receptors in a healthy skin have
documented their expression mainly in keratinocytes of spinous and granular layers, to a lower extent in the basal layer, in melanocytes and in blood vessels (16, 55).

In the presented results, immunohistochemical detection in paraffin sections of melanoma demonstrated a high level of MT1 expression. In the case of primary tumours, the level of MT1 receptor expression was higher than in the examined lymph nodes. The membranous-cytoplasmic MT1 expression was likely due to binding of the antibodies to the second cytoplasmic domain of MT1 membrane receptor, as suggested by other authors (37, 49).

Statistical analysis allowed us to verify that MT1 expression was positively correlated with the depth of tumour infiltration. The earlier literature data also documented the positive correlation between expression of MT1 receptors and grade of malignancy in mammary gland cancer (49). Some authors have pointed to an inverse relationship between blood melatonin concentration and the level of MT1 expression (56). A probability exists that the documented high expression of MT1 receptors in highly advanced (grade III) melanomas reflects low melatonin level in the patient.

In the studied cases, expression of Ki-67 antigen was proven to be low. In majority of the cases of the primary tumour presence of the protein was demonstrated in less than 5% of cells. In primary tumours, a positive reaction was noted on average in 7.8% cells. In lymph nodes, the positive cells were twofold more numerous, thus confirming the rule that metastatic cells are biologically much more active and tend to divide. In the studied cases, Ki-67 antigen expression also had a strong correlation with the depth of tumour infiltration. Our results have corroborated the literature data (57).

In our study we have also undertaken an attempt to examine potential relationship between expression of melatonin MT1 receptors and the level of Ki-67 antigen expression. Preliminary results have suggested an inverse relationship between the two variables, both in primary tumours and in lymph nodes. The available references contain no data on the relationship in malignant melanoma, which makes interpretation of our result difficult.

Several ambiguities exist on the functioning of melatonin MT1 receptors in melanomas. Most of the available reports on MT1 receptors are based on studies on various cell lines but no immunohistochemical results have been given, particularly as related to skin melanoma (33, 36, 46, 53). On several occasions and using multiple cells lines of melanoma the inhibitory effect of melatonin has been demonstrated to develop via mediation of its receptors (36, 37, 49). However, causes of the high plasticity of the receptors remain mostly unknown. Numerous literature data suggest that expression of MT1 receptor on cells of malignant melanoma increases efficacy of the anti-proliferative effect of melatonin (24). Moreover a positive effect of melatonin has been demonstrated on the outcome of chemotherapy of certain tumours, including melanoma (7). The results of studies on melatonin and its receptors published so far are encouraging and demonstrate a potential to open up new, alternative therapeutic approaches in treatment of dermal cancer and of other tumours. Results obtained in this study on the expression of MT1 receptor in dermal melanoma and in metastatic lymph nodes may promote further experiments and lead to a better understanding of the receptor’s role in tumours of this type. The positive correlation of MT1 expression with the depth of tumour infiltration and expression of Ki-67 antigen may point to the potential of using expression of the receptors for prognostic appraisal of melanoma.

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