Immunohistochemical Evaluation of Metallothionein, Mcm-2 and Ki-67 Antigen Expression in Tumors of the Adrenal Cortex

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Abstract. *Background: The aim of this study was to assess the metallothionein (MT), maintenance protein 2 (Mcm-2) and Ki-67 expressions in adrenocortical adenomas and carcinomas in comparison to normal tissue and evaluate the correlations between these markers of proliferation and between these markers and tumor diameter. Materials and Methods: The expression of MT, Mcm-2 and Ki-67 was assessed by immunochemistry in forty-eight adrenocortical adenomas, six adrenocortical carcinomas and eleven normal adrenal cortex tissue samples. Results: The expressions of MT, Mcm-2 and Ki-67 in the adrenocortical carcinomas were significantly higher than in the adenomas and normal tissue (p<0.05). The levels of Mcm-2 were also higher in the adrenocortical adenomas compared to the normal tissue (p<0.05). The Mcm-2 expression showed a positive correlation to the expression of MT in the adrenocortical carcinomas (r=0.773; p<0.05) and to the expression of Ki-67 in the adrenocortical adenomas (r=0.432; p<0.05). The malignant tumor diameter was positively correlated with the MT and Mcm-2 expressions (r=0.766, p<0.05 and r=0.620, p<0.05, respectively). Conclusion: The assessment of Mcm-2 expression seems to be of special importance as a marker of adrenocortical dysplasia and a reliable indicator of malignancy in suspicious masses of the adrenal cortex.*

Tumors of the adrenal cortex most often derive from the epithelial cells of the adrenal cortex. Although these

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neoplasms show variable hormonal activity, they usually remain clinically 'silent'. Since they are discovered incidentally with various imaging techniques, they are referred to as 'incidentaloma' (1).

With the increasing quality and frequency of imaging examinations, the incidentaloma has become a common finding present in up to 4% of patients examined for non-adrenal reasons. The data from autopsies suggest nearly twice as high prevalence, reaching 7% (2). On the other hand, malignant tumors of the adrenal cortex, that is adrenocortical carcinomas, are rare neoplasms: their incidence does not exceed 1/1,000,000/year, accounting for 3% of all endocrine neoplasms (3).

The macroscopic, microscopic and clinical characteristics of lesions are particularly helpful for the differential diagnosis between benign and malignant lesions. The most widely used systems of differentiating between carcinoma and adenoma were developed by Hough *et al*., van Slooten *et al*., Weiss *et al*. and Aubert *et al*. (4-7). In clinical practice, the endocrine activity of the tumor is of the greatest importance because it qualifies the patient for an adrenalectomy. Nevertheless, malignant lesions often secrete no hormones at all, so in the majority of surgical departments the most important indication for surgical treatment is the tumor diameter. This practice stems from the fact that 10% of lesions exceeding 4 cm are malignant and the risk of malignancy reaches as much as 25%-98% in tumors bigger than 6 cm (3, 8). However, malignant lesions may be as small as 1.7 cm so the diameter criterion seems insufficiently accurate (9).

The differential diagnosis between malignant and benign lesions not only determines surgical treatment, but also strongly influences postoperative patient management, in terms of the frequency of follow-up, the introduction of chemotherapy or the administration of mitotane. While karyotype analysis seems to be irrelevant for differentiation,

immunohistochemical assays of tissue sections and cellular specimens obtained with fine-needle aspirations are particularly useful in differentiating adrenocortical carcinomas from renal cells and metastases originating from the liver and from the adrenal medulla (10). The immunoreactivity for inhibin alpha and A-103 is a sensitive but unspecific marker of adrenocortical carcinomas (11, 12). Specific antibodies against Ad4BP/SF-1 (adrenal-4 binding protein/steroidogenic factor-1) have also shown clinical relevance by identifying cells of the adrenal cortex (13). Adrenocortical carcinomas have been found to be either negative or weakly positive in cytokeratin testing and assays revealing the presence of the membrane epithelial antigen, carcinoembryonal antigen and glycoprotein HMFG-2 were all negative (14). Furthermore, carcinomas of the adrenal cortex are negative for the presence of the chromogranin A, which is the most reliable marker used for differentiating them from neuroendocrine tumors developing from the adrenal medulla (15). Immunohistochemical methods have also allowed the discovery of new features of malignancy, such as the increased expression of various markers of proliferation described below (16).

Metallothioneins (MTs) are low molecular weight proteins that play a significant role in cellular homeostasis (17). The high cystein content permits the binding of various metal ions, such as mercury, cadmium, silver and platinum, however only Zn and Cu ion binding is of physiological importance. Studies have indicated that the MTs act as cellular scavengers of reactive oxygen species and that they buffer toxic heavy metals ions (18, 19). In addition, the MTs are involved in cellular proliferation and differentiation by maintaining constant concentrations of Zn and Cu ions, which means that the MTs play an important role in the process of carcinogenesis (17, 20). Immunohistochemical assays have shown higher MT expression in the cells of various tumors, such as cancer of the breast, ovary, uterus, kidney, urinary bladder, esophagus, stomach, pancreas and colon (21-29). MT expression characterizes cells with a high mitotic rate and positively correlated with the grade of malignancy as well as with the proliferation index based on the Ki-67 expression and proliferating cell nuclear antigen (PCNA) (28, 30).

The minichromosome maintenance protein 2 (Mcm-2), also known as CDCL1 and BM28, belongs to a heterogeneous group of ten nuclear proteins involved in DNA synthesis (31). Mutation of the Mcm-2 gene (locus 3q21) is associated with acute myeloid leukemia (31). A heterohexamer of Mcm-2, – 7 takes part in DNA replication as a part of the replication fork (32). These proteins are bound to chromatin which ensures that DNA replication occurs only once in a cell cycle (33). The Mcm proteins are not detected in cells in G0-phase, but only in those undergoing mitosis, thus their higher expression should characterize neoplasms (34). Nevertheless, according to

some studies, high levels of Mcm-2 in a tumor did not differentiate between benign and malignant lesions (35).

Ki-67 is a non-histonic protein of the nuclear matrix. With its expression detected in the G_1-, S-, G_2- and M-phases of the cell cycle, it is thought to be involved in DNA replication. Zheng et al. demonstrated that knockout of the Ki-67 gene led to inhibition of cancer cells and increased their apoptotic rate (36, 37). Furthermore, high Ki-67 expression has been found in tumors of the breast, lung and brain, an observation that has made this protein a valuable proliferation marker (17, 38-40). However, other studies have provided contradictory results, where no correlation was found between the malignancy grade (in the uterine cervix and prostate) and the expression of Ki-67 (38, 41, 42).

The present study aimed at comparing the expressions of metallothionein, Mcm-2 and Ki-67 in adrenocortical carcinomas and benign tumors as well as in normal tissue of the adrenal cortex. The correlation between the tumor (both malignant and benign) diameter and the expression levels of the proliferation markers was also evaluated.

Materials and Methods

Patients. The material for the study originated from 54 patients who underwent surgery in the Second Department of General and Oncologic Surgery of the Medical University in Wroclaw in the years 1998-2005. Forty-eight benign tumors, all diagnosed as adenoma corticis suprarenalis, were obtained from 35 women (73%) and 13 men (27%) aged 23-76 years (mean age 52 years). The mean diameter of the benign lesions was 27 millimeters. Six malignant tumors, all diagnosed as carcinoma corticis suprarenalis, were excised from 3 women and 3 men aged 50-70 years (mean age 62 years). The mean diameter of the malignant lesions was 63 millimeters. Eleven archived tissue samples normal adrenal cortex originating from 3 (27%) women and 8 (73%) men (aged 20-71 years; mean age 48 years) were provided by the Department of Histology and Embryology of the Medical University in Wroclaw.

Tissue analysis. Biopsies of the six adrenocortical carcinomas and forty-eight adenomas and the eleven normal adrenal cortex samples were fixed in 10% buffered formalin, dehydrated and embedded in paraffin blocks. The immunohistochemical reactions were performed on the paraffin sections and seven 4 μm-thick sections were cut from each sample. Three sections were stained for the detection of Mcm-2, MT or Ki-67 expression, one section was stained with hematoxylin and eosin for traditional light microscopy and the other three served as negative controls. Mouse monoclonal antibodies specific to Ki-67 (clone MIB-1 diluted 1:100; DakoCytomation, Denmark), MT (clone E9 diluted 1:100; DakoCytomation) and Mcm-2 (clone CRCT2.1 diluted 1:50; Novocastra, UK) were used. Negative control reactions accompanied all the above reactions and the specific antibodies were substituted with a primary negative control (DakoCytomation). The paraffin sections used to detect Ki-67 and Mcm-2 were boiled in antigen retrieval solution (DakoCytomation) in a microwave oven for 20 minutes for antigen retrieve. The visualization of the

immunohistochemical reaction was obtained with LSAB+ System HRP (labeled streptavidin biotin with horse-radish perdoxidase; DakoCytomation) and diaminobenzidine (DAB).

Coded preparations were independently evaluated by two pathologists. The evaluation of Ki-67 and Mcm-2 was based on the percentage of cells with a nuclear color reaction. The results were recorded on a five-point scale, where 0 reflected no reaction; 1, corresponded to 1% - 10% of cells with a positive reaction; 2, 11% to 25%; 3, 26% to 50%; and 4 points to more than half of the cells positively stained (17).

The evaluation of MT expression was based on a semi-quantitative immunoreactivity scores (IRS) approach that took into account the percentage of positively stained cells (value A: 0 points, no cells with positive reaction; 1, fewer than 10% of cells stained positively; 2, 10% to 50% of positive cells; 3, 51% to 80% of positive cells; and 4, more than 80% of cells positively stained) and the intensity of the color reaction (value B: 0, no reaction; 1, weak reaction; 2, moderate reaction; and 3, strong reaction). The presented results are given as the product of all the points (value C=AxB) given for the individual scores, resulting in total values ranging from 0 to 12 points, where a weak reaction ranges from 0 to 4 points and a strong reaction from 6 to 12 points (43).

Statistical analysis. Statistical analysis was carried out using the Statistica 5.1 PL software (StatSoft, Cracow, Poland) with Mann-Whitney *U*-test for the analysis of the differences in expression levels of the studied markers and Pearson's correlation analysis to study the correlation of marker expression in both malignant and benign lesions. All the results were considered statistically significant for $p < 0.05$.

Results

Table I summarizes the results of immunostaining for the three antigens and presents their levels of expression in the healthy tissue samples, adrenocortical adenomas and adrenocortical carcinomas.

In the control samples, the expression of metallothionein was scattered nuclear and cytoplasmic (Figure 1A). Expression limited to the nuclei only characterized the benign lesions (Figure 1B), while the expression was observed to be both nuclear and cytoplasmic in the adrenocortical carcinomas (Figure 1C). The difference in MT expression between the adrenocortical carcinomas and adenomas proved to be statistically significant ($p < 0.05$).

Staining for Ki-67 revealed hardly any expression in the control tissues (Figure 1D), a faint level of expression in the adenomas (Figure 1E) and strong nuclear expression in the adrenocortical malignant tumors (Figure 1F). The difference in Ki-67 expression between the adrenocortical carcinomas and adenomas proved to be statistically significant ($p < 0.05$).

The control samples showed no expression of Mcm-2 (Figure 1G). This protein, however, was expressed in the cytoplasm of both benign and malignant adrenocortical tumors (Figure 1H and 1I). The differences in Mcm-2 expression between the adrenocortical adenomas and the

Table I. *Mean expression values (± standard deviation) of the antigens in control tissue samples, adenomas and carcinomas of the adrenal cortex.*

Sample	Antigen		
	MT	Ki-67	Mcm-2
Control	4.00±1.48	0.09±0.30	0.09±0.30
Adenoma	4.71±2.16	0.52±0.54	0.88**±0.89
Carcinoma	6.67*±3.61	1.83*±1.47	2.17*±1.17

*Expression statistically higher in the malignant lesions than in the benign tumors and the control tissue, $p < 0.05$; **expression statistically higher in the adenomas compared to the control samples, $p < 0.05$.

normal tissue and between the adrenocortical carcinomas and the adenomas proved to be statistically significant ($p < 0.05$).

A very high positive correlation was observed between the MT and Mcm-2 expressions in the adrenocortical carcinomas (r=0.773) (Figure 2). However, only a low positive correlation was found between the expression of Ki-67 and Mcm-2 in the malignant tumors (r=0.136) and the correlation between MT and Ki-67 expressions was found to be intermediate negative (r=–0.351).

In the adrenocortical adenomas, moderate positive correlation was found between the Ki-67 and Mcm-2 expressions (r=0.432) (Figure 3). The MT and Ki-67 expressions showed weak positive correlation (r=0.134). No correlation was observed between the expression of MT and Mcm-2 (r=0.095).

In the adrenocortical adenomas, no correlation was observed between the tumor diameter and the expression of Ki-67 and Mcm-2 (r=0.071 and 0.079 respectively), a low negative correlation was noted between the tumor diameter and the expression of MT (r=–0.296).

Very high positive correlation linked the expression of MT and the diameter of the malignant lesions (r=0.766) (Figure 4). The expression of Mcm-2 was found to be highly related to the carcinoma diameter (r=0.620). No correlation was observed between the Ki-67 expression and the diameter of the carcinoma samples (r=–0.045).

A combined analysis of the correlation between the tumor diameter (both adrenocortical adenomas and carcinomas) and the expressions of the studied markers revealed a moderate positive correlation for Ki-67 and Mcm-2 (r=0.428 and 0.395 respectively) and a low one for MT (r=0.105).

Discussion

The mitotic rate, tumor diameter and the expression of the Ki-67 protein are currently the most relevant prognostic and pathological markers of adrenocortical carcinoma (6, 44).
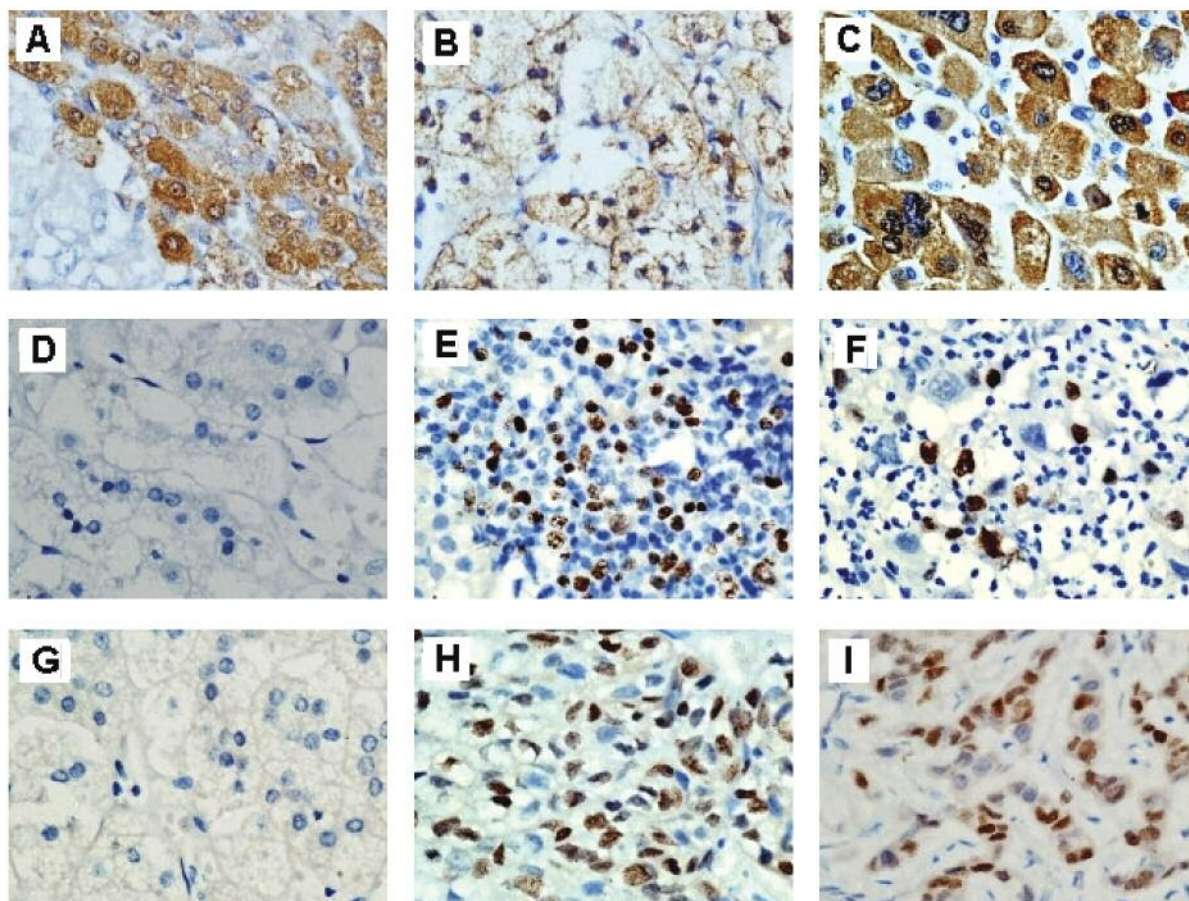
Figure 1. *Expression of MT: moderate in healthy tissue (A), low in adrenocortical adenoma (B) and high in adrenocortical carcinoma (C). Expression of Ki-67: absent in healthy tissue (D), high in adrenocortical adenoma (E) and moderate in adrenocortical carcinoma (F). Expression of Mcm-2: absent in healthy tissue (G), high in adrenocortical adenoma (H) and high in adrenocortical carcinoma (I). Immunohistochemical reaction, counterstained with hematoxylin. Magnification ×400.*

To our best knowledge, to date there have been no studies on the role of MT in adrenocortical tumors. In the present study MT expression was high in the adrenocortical carcinomas in agreement with other tumor studies (15, 21-29, 45-47). MT has also been found to be positively correlated with other proliferation markers such as Ki-67 in various tumors (17, 29). On the other hand, some studies showed no correlation between MT and Ki-67 expressions in laryngeal carcinomas and colorectal tumors (23, 47). The present results, in contrast to these studies, supported the observation that MTs are co-expressed in malignant lesions with Ki-67, but in view of the results of Pastuszewski *et al.* (47) and our small cancer case group, the predictive value of MT in adrenocortical carcinomas should be re-assessed. The high positive correlation found in this study between the MT expression and the diameter of the adrenocortical malignant tumors may enable immunohistochemical Gleason score tumor grading (G) and may be useful as a

prognostic factor for incidentalomas. However, this observation should be confirmed by further studies because MTs in laryngeal carcinomas were proven to have no influence on the tumor aggressiveness and speed of progression of the disease (47).

Ki-67 is apparently a valuable prognostic factor only in some neoplasms. However, in adrenocortical tumors, the Ki-67 expression along with the histopathological features have been found to be the most reliable characteristics in discerning malignancy of the lesions (48-50) and the usefulness of assessing the Ki-67 expression for differentiating between benign and malignant lesions was confirmed in the present study. Because only few cases of adrenocortical carcinoma were available for this study, further research of more specimens is needed.

Increased Mcm-2 expression characterizes various neoplasms such as those of the oral cavity, salivary glands, esophagus, thyroid, lung and urinary bladder (51-56),
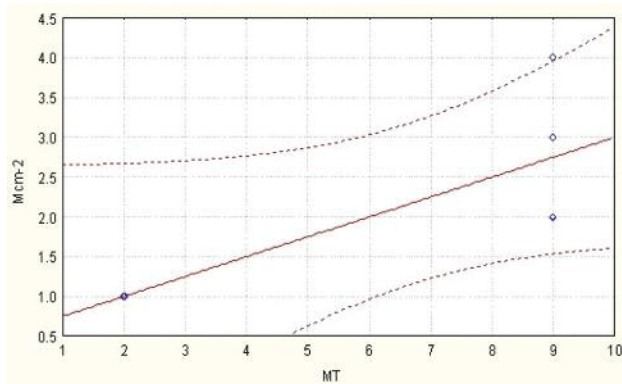
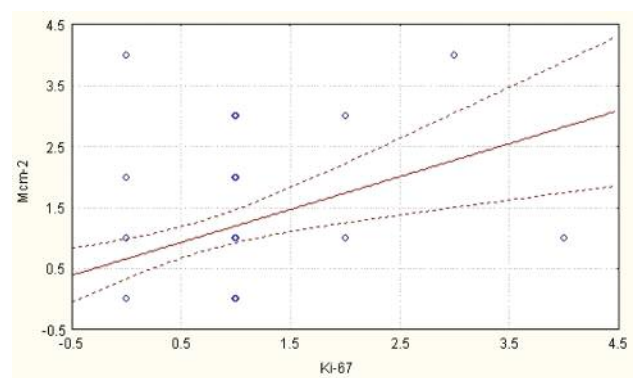Figure 2. *Correlation of MT expression with the expression of Mcm-2 in adrenocortical carcinoma. r=0.773, p<0.05.*

Figure 3. *Correlation of the Ki-67 expression with the expression of Mcm-2 in adrenocortical adenomas. r=0.432, p<0.05.*

although it seems not to be specific for endometrial carcinomas (57). Despite being a recognized marker of cellular proliferation, to our best knowledge, to date its role in adrenocortical tumors has not been studied. Furthermore, some researchers have indicated that the increased expression of Mcm-2 is a reliable marker of dysplasia in Barrett's esophagus and high-risk normal prostate epithelium (52, 58). The significantly higher expression, of Mcm-2 in the adrenocortical adenomas as compared to the control tissue in the present study suggested a similar conclusion and thus advocates the radical treatment of all adrenocortical masses with high Mcm-2 expression in agreement with other authors who have suggested the radical treatment of all, even the smallest, incidentally discovered adrenocortical tumors. Moreover, the higher Mcm-2 expression in the malignant lesions than the benign ones supported the conclusion of Vargas *et al*. (55) that the assessment of Mcm-2 levels allowed the differentiation of carcinomas from adenomas in tumors of the salivary glands.

The evaluation of the MT, Ki-67 and Mcm-2 expressions and their mutual correlations may allow an accurate prognosis and better management of patients with incidentalomas. The correlation between the expressions of Ki-67 and Mcm-2 and both benign and malignant tumor diameters suggests that such evaluation should become a routine practice in the clinical assessment of all adrenocortical tumors.

Conclusion

The expression of MT, Mcm-2 and Ki-67 is significantly higher in adrenocortical carcinomas in comparison to adrenocortical adenomas and normal controls. Mcm-2 expression seems to be of special importance as a marker of adrenocortical dysplasia and a reliable indicator of malignancy in suspicious masses of the adrenal cortex.
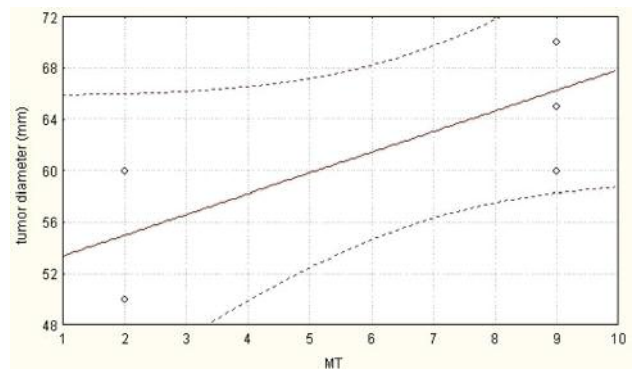

Figure 4. *Correlation of MT expression with the tumor diameter in adrenocortical carcinomas. r=0.766, p<0.05.*

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