

P R A C E O R Y G I N A L N E
ginekologia

Increase of nuclear expression of metallothionein I/II in neoplastic transformation of the endometrium

Wzrost jądrowej ekspresji metalotioneiny I/II w transformacji nowotworowej endometrium

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Abstract

Objectives: The aim of our study was to investigate the expression of epidermal growth factor receptor (EGFR), metallothionein (MT) I/II, and Ki-67 antigen in endometrial cancer. We analyzed cytoplasmic (cMT) and nuclear (nMT) metallothionein fractions separately. Moreover, we evaluated the relationships between expressions of the above mentioned proteins and compared them with clinicopathologic data.

Material and methods: The study material included paraffin-embedded endometrial cancer samples from 84 patients. The control group consisted of 52 non-neoplastic endometrium samples. Immunohistochemical reactions were performed using monoclonal antibodies against EGFR, MT I/II and Ki-67. Expression intensity of the tested proteins was assessed by computer image analysis software. Chi-square, Spearman's correlation, Mann-Whitney and Kruskal-Wallis tests were used for statistical analysis with Statistica 8.0 PL.

Results: Strong expression of nMT was revealed in endometrial cancer cells in relation to benign hyperplasia ($p < 0.001$) and normal cells ($p < 0.001$) of the endometrium. Statistically significant but weaker expressions in analogous relationships were observed for cMT. Moreover, higher grade of histological malignancy G was positively associated with increased expression of nMT ($p = 0.009$).

Conclusions: Expression of nMT remains in distinct correlation with neoplastic transformation of the endometrium and histologic grades. Our results clearly indicate a need for further research on metallothionein expression in tumor cells.

Key words: **endometrial cancer / epidermal growth factor receptor / EGFR / metallothionein /**

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Streszczenie

Cel pracy: Nasze badania dotyczyły analizy ekspresji receptora naskórkowego czynnika wzrostu (EGFR), metalotioneiny (MT) I/II oraz antygenu Ki-67 w raku endometrium. Nasilenie ekspresji metalotioneiny analizowaliśmy oddzielnie we frakcjach cytoplazmatycznej (cMT) oraz jądrowej (nMT). Ocenie zostały poddane wzajemne zależności pomiędzy ekspresją badanych białek jak również ich ekspresja w odniesieniu do danych kliniczno-patologicznych.

Materiał i metody: Materiał do badań stanowiły 84 archiwalne bloczki parafinowe raków trzonu macicy. Grupa kontrolna składała się z 52 przypadków nienowotworowych zmian endometrium. Reakcje immunohistochemiczne przeprowadzono przy użyciu przeciwciał monoklonalnych przeciwko: EGFR, MT I/II oraz Ki-67. Analizę intensywności ekspresji badanych białek przeprowadzono z wykorzystaniem oprogramowania do komputerowej analizy obrazu. Obliczenia statystyczne zostały przeprowadzone za pomocą testów: chi-kwadrat, korelacji Spearmana, Manna-Whitneya i Kruskala-Wallisa, przy użyciu programu Statistica 8.0 PL.

Wyniki: Zaobserwowano silniejszą ekspresję nMT w komórkach raka endometrium w stosunku do łagodnego wzrostu ($p < 0,001$) oraz komórek prawidłowego endometrium ($p < 0,001$). Analogiczne zależności były obserwowane w przypadku ekspresji cMT, jednakże różnice były wyraźnie mniejsze. Wykazano ponadto, że wraz ze wzrostem stopnia histologicznej złośliwości G, rośnie również ekspresja nMT ($p = 0,009$).

Wnioski: Wzrost ekspresji nMT silnie koreluje z transformacją nowotworową błony śluzowej trzonu macicy oraz rosnącym stopniem złośliwości histologicznej. Uzyskane wyniki wyraźnie wskazują na konieczność dalszych badań nad ekspresją metalotioneiny w raku endometrium.

Słowa kluczowe: rak endometrium / receptor naskórkowego czynnika wzrostu /
/ EGFR / metalotioneina /

Introduction

Endometrial cancer is the most frequent gynecologic malignancy in developed countries. Globally, it is the seventh most common malignant neoplasm in women, with the annual rates of incidence and cause of death estimated at approximately 142 000 and 42 000 women, respectively [1, 2]. The neoplasm originates from glandular cells of the epithelium, what constitutes a typical presentation of adenocarcinoma. Histologically, three general subtypes of endometrial adenocarcinomas are distinguished: endometrioid (80% of cases), serous (5-10%) and clear cell (5%) *endometrial carcinoma* [3, 4]. According to a study published by Bokhman in 1983, endometrial cancers can be divided into two types from the standpoint of pathogenesis [5], i.e. type I: estrogen-dependent (referring to endometrioid subtype), and type II: estrogen-independent (referring to serous and clear cell subtype). Risk factors for type I tumors are associated with hyperestrogenic states and are characterized by relatively lower aggressiveness, with a generally successful outcome. Type II risk factors are not defined, as it has been shown that they derive from the atrophic epithelium. They are characterized by an aggressive clinical course and have unfavorable prognosis [6, 7].

Nowadays, early diagnosis and an increasing number of therapeutic options are strongly emphasized in oncologic treatment. The search for new markers of prognostic and predictive value continues, as well as determination of the expression of some proteins currently used in cancer immunotherapy. In our study, we attempted to determine the potential use of epidermal growth factor receptor and metallothionein with regard to the commonly used proliferative marker Ki-67 in therapeutic and diagnostic management of endometrial carcinoma.

Epidermal growth factor receptor (EGFR) is a cell-surface receptor for the members of the epidermal growth factors family, which play an important role in the regulation of cell growth, proliferation, and differentiation [8].

While they regulate the mentioned processes, their overexpression or overactivity may be observed in carcinogenesis. Mutations, amplifications or misregulations of EGFR or its family members are implicated in about 30% of all epithelial neoplasms [9, 10]. Apart from the suggested usage of this protein as a marker of proliferation in some types of neoplasms, i.e. lung and colon cancer or glioblastoma multiforme, the EGFR expression assay constitutes an important element of the oncologic treatment. Monoclonal antibody inhibitors are aimed at EGFR for termination of uncontrolled cell division – typical for cancer disease [11, 12].

Metallothioneins (MT) are a group of low-molecular weight and cysteine-rich proteins represented by four isoforms. Their function consists in binding a wide range of metals and controlling harmful intracellular stimuli, such as metal exposure, oxidative stress, glucocorticoids and hydric stress [13]. MT-I and MT-II isoforms are present in most tissues in adult mammals. They occur in both, healthy and neoplastic tissues. Expression of these proteins can be observed in the nucleus (nMT) and cytoplasm (cMT) of cells. Numerous studies demonstrated their overexpression in actively proliferating cells, especially neoplastic ones [14, 15]. Additionally, various studies revealed prognostic significance of metallothionein expression in many types of neoplasms [16].

Ki-67 is a nuclear protein essential for cellular proliferation. The fraction of neoplastic cells expressing Ki-67 (the *Ki-67 labeling index*) is often compared with the clinical course of the disease. Despite Ki-67 being used routinely as a proliferation marker, various authors have reported divergent results concerning its prognostic and predictive value in different types of cancers [17]. Therefore, other markers of malignant diseases such as endometrial cancer remain to be found.

Objectives

Due to high incidence of endometrial cancer and noticeable lack of reliable prognostic and predictive markers, further studies are necessary. We analyzed the expression of the investigated proteins: EGFR, cMT, nMT with regard to Ki-67 antigen in endometrial cancer. Moreover, we investigated the relationships between these proteins and compared them with clinicopathologic data.

Material and methods

Patients

The study was performed on 84 archival paraffin-embedded samples of endometrioid type of endometrial cancer (I group). The control group consisted of 52 cases of non-neoplastic endometrium samples (28 cases of normal endometrium (II group) and 24 cases of benign endometrial hyperplasia (III group)). Neoplastic and non-neoplastic samples with clinical and pathological data were obtained from the First Department of Gynecology and Obstetrics, Wrocław Medical University (Table I). Local Ethics Committee approved of the study. Histological malignancy grade (grading, G) and clinical advancement (staging, S) were determined using guidelines suggested by FIGO in 2009 [18].

Immunohistochemistry (IHC)

Paraffin blocks were cut to sections of appropriate thickness of 7 μm (histological examination) or 4 μm (immunohistochemical reactions). Immunohistochemistry was performed on paraffin sections mounted on silanized slides (Dako Cytomation, Glostrup, Denmark). Deparaffinization and antigen retrieval were performed in Target Retrieval Solution, pH 6 (96°C, 20 min.) for Ki-67 and MT I/II and by Proteinase K (95°C, 20 min) for EGFR. Activity of endogenous peroxidase was blocked by a 10 min-exposure to 3% H_2O_2 . The sections were then washed in tris-buffered saline (TBS) and incubated with primary antibodies: anti-Ki-67 (clone MIB-1, 1:150), anti-EGFR (clone H11, 1:25) and anti-MT I/II (clone E9, 1:200) on neoplastic and non-neoplastic endometrial samples. All reactions were accompanied by positive and negative control reactions. Negative control was carried out using N-Series Universal Negative Control Mouse, whereas positive was made on tissues suggested by the supplier, *i.e.* tonsils for Ki-67, skin for EGFR, and ovarian cancer for MT I/II. EnVision FLEX and 3,3-diaminobenzidine (DAB) were used for visualization of the investigated antigens, in accordance with the manufacturer's instructions. All slides were counterstained with Mayer's hematoxylin. All reagents were obtained from DakoCytomation.

Evaluation of the intensity of IHC reaction

Different methods of evaluation were used due to different cellular localization of the tested markers. Routine techniques were utilized for quantification of immunohistochemical reactions. Nuclear expression of Ki-67 antigen was evaluated in five fields, with the highest number of cancer cells yielding a positive reaction (hot spots). The percentage of positive cells in each hot spot was evaluated by scoring the brown-labeled cell nuclei of glandular cells to all glandular cells (stained and unstained). The general result for every sample was an average of the five hot spot percentages. Cytoplasmic and membranous

expression of EGFR was evaluated using a four gradual quality scale (from 0 to +++), where 0 defines no reaction, "+" weak, "++" moderate and "+++" strong reaction. Expression of cMT was evaluated with the use of the immunoreactive semi-quantitative (IRS) method, according to Remmele and Stegner [19]. This technique takes into account the intensity of the color reaction and the percentage of the positive cells. The final score represents the result of points given for individual traits and range between 0 and 12 (Table II). Expression of nMT was evaluated with the use of the semi-quantitative five gradual scale (from 0 to 4), depending on the percentage of cells manifesting nuclear color reaction, according to the section listed in Table II ("Percentage of positive cells"). Evaluation included only the glandular cells, excluding the stroma. All slides were scored using BX-42 light microscope assisted by computer image analysis software (Olympus, IMARIS – Imaging Software for Life Sciences Microscopy; Tokyo, Japan). The intensity of the IHC reactions in coded preparations was independently evaluated by two researchers (LJ, MJ) blinded to clinical data of the respective patients. Moreover, re-evaluation with a double-headed microscope was performed in doubtful cases until a consensus was achieved.

Statistical analysis

The obtained results were statistically analyzed using Statistica 8.0 PL (Statsoft, Krakow, Poland). The relationships between the expression of EGFR, MT I/II and Ki-67 were examined using Spearman's rank correlation test. The analysis of frequency was carried out using the chi-square test with categorical distribution. The relationship between expression intensity of markers and histological malignancy grade (G) and clinical advancement (S) was examined by ANOVA rank test of the Kruskal-Wallis and Mann-Whitney tests. Overall survival (OS) was determined by the Kaplan-Meier method and the significance of differences was determined by the log-rank test. In all analyses, results were considered statistically significant at $p < 0.05$.

Results

Statistical analysis was conducted in three groups of cases: I – cancer samples, II – normal endometrium, and III – benign endometrial hyperplasia. All possible relationships between the analyzed markers and clinicopathologic data were tested, but only those reaching the level of statistical significance and relevant for further analysis are shown below.

Expression analysis

Nuclear and cytoplasmic patterns of MT I/II expression are presented in Figure 1 and Figure 2, respectively.

A strong positive correlation was revealed between the expression of nMT and cMT in group I ($r=0.63$), group II ($r=0.86$), group III ($r=0.70$), and combined groups II+III ($r=0.81$), and groups I+II+III ($r=0.69$) ($p < 0.05$ for all; Spearman test). Moreover, a correlation between the expression of nMT and EGFR in the combined groups II+III ($r=-0.29$) and nMT and Ki-67 in groups I+II+III ($r=0.17$) was observed ($p < 0.05$ for both; Spearman test). Additionally, the expressions of nMT with Ki-67 in group I ($r=0.25$) and cMT with Ki-67 in group III ($r=-0.40$) correlated at the level of significance ($p=0.05$ for both; Spearman test).

Table I. Clinical and pathological characteristics of the studied patients.

Clinical/pathological parameter	N (%)
Mean age in study group (range)	61.42 (40-83)
Mean age in control group (range)	51.40 (32-72)
Menopausal status	
Study group PRE	17/84 (20.3%)
POST	67/84 (79.7%)
Control group PRE	18/84 (21.4%)
POST	34/84 (78.6%)
Histological malignancy grade (G)	
G1	55/84 (65.4%)
G2	20/84 (23.8%)
G3	9/84 (10.8%)
Clinical advancement (S)	
I A	39/84 (46.4%)
I B	35/84 (41.6%)
II-IVB	10/84 (12.0%)

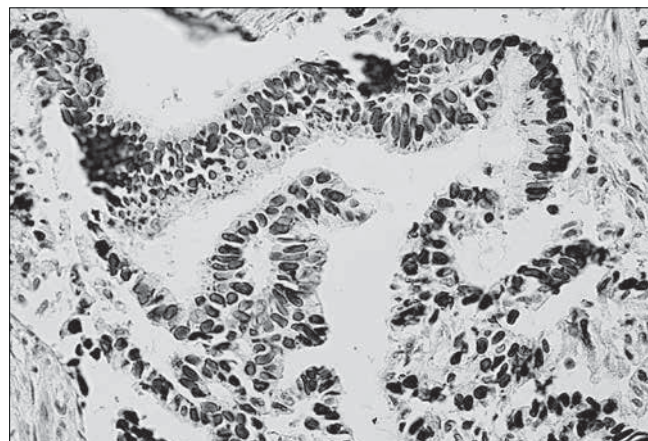
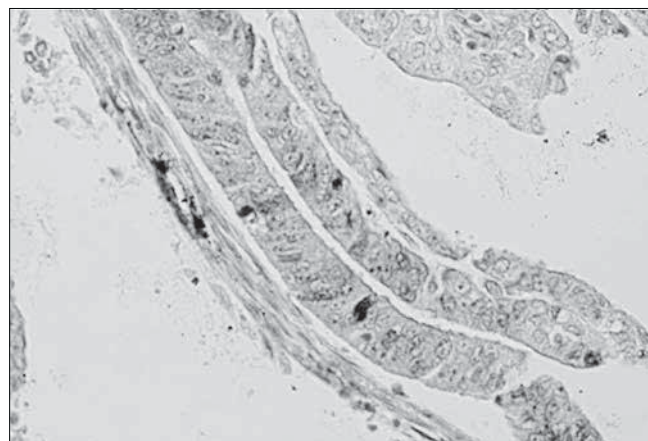
Table II. Evaluation of MT expression using IRS scale according to Remmele and Stegner.

Percentage of positive cells	Points	Intensity of color reaction	Points
no positive reaction	0	no color	0
< 10% positive cells	1	weak color	1
10-50% positive cells	2	moderate color	2
50-80% positive cells	3	strong color	3
> 80% positive cells	4		

Significant differences in the expression of the analyzed markers were assessed by Mann-Whitney test. Relevant differences were found for cMT expression between group I (77.8% of positive cells) vs. group II (35.7%) ($p=0.027$) and group I vs. the combined groups II+III (42.3%) ($p=0.018$); in nMT expression between group I (81.8%) vs. group II (32.1%), group I vs. group III (37.5%), and group I vs. the combined groups II+III (34.6%) ($p<0.001$ for all), as well as in EGFR expression between group I (34.6%) vs. group II (71.4%), group I vs. group III (70.8%), and group I vs. the combined groups II+III (71.2%) ($p<0.001$ for all). No statistically significant differences in Ki-67 expressions between the tested groups were observed.

Clinicopathologic data analysis

Expression of all tested markers in relation to clinicopathologic data (age, menopausal status, grading and staging) revealed statistically significant correlation only between the level of nMT expression and grade of histological malignancy (Kruskal-Wallis test $p=0.008$ and chi-square test $p=0.006$). Increase of G was positively associated with increased expression of nMT (G1 – 77.1%, G2 – 85% and G3 – 99.9% of cells). Moreover, the survival analysis did not show any statistically significant results for all tested markers.

**Figure 1.** Nuclear expression of metallothionein I/II in endometrial cancer. 400x magnification.**Figure 2.** Cytoplasmic expression of metallothionein I/II in endometrial cancer. 400x magnification.

Discussion

Due to high incidence of endometrial cancer, it is important to search for new prognostic and predictive markers. The commonly used Ki-67 antigen is often a point of reference for the tested markers. We also used it to compare the expression of the studied proteins. First of all, because their expression was also demonstrated in the proliferating cells, as well as the fact that the literature offers very scant information in this subject. Additionally, we verified the expression profile of MT I/II and EGFR with regard to clinicopathologic data.

Expression of MT was analyzed in many histological and molecular studies concerning different types of cancer. To the best of our knowledge, only four experiments concerning MT expression in endometrial cancer have been reported so far in the literature. McCluggage et al., observed strong expression of MT in neoplastic cells and a positive association of its expression with an increase of G [20]. Ioachim et al., compared MT expression in normal endometrial tissue, benign hyperplasia, and cancer cells. They obtained a statistically higher levels of MT expression in the cancer group and a positive association of MT expression with increase of G. No relationship between MT expression and clinical advancement of the disease was revealed [21].

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In view of contradictory data on MT function in carcinogenesis, Pedersen et al., investigated the role of MT and determined it to be variable and dependent on many additional factors, such as intracellular localization of the protein [22]. Supporting conclusions were published by Levadoux-Martin et al., in 2001, who demonstrated that nuclear MT fraction has anti-apoptotic and protective effects against DNA [23]. This supports the hypothesis that nuclear MT overexpression in neoplastic cells may be associated with their increased survival and resistance to external factors, including radio- and chemotherapy. McCluggage and Ioachim evaluated MT expression without distinction into fractions – nuclear and cytoplasmic. In the available literature, we found only one study which analyzed them separately. A study conducted by Wicherek et al., analyzed a small group of 13 cancer cases. They observed similar cMT expression in normal and neoplastic endometrium, while nMT expression was significantly stronger in the cancer group [24].

In light of the above data, we decided to assess both MT fractions, separately. In our study, cMT and nMT expression was stronger in the endometrial cancer group as compared to control subgroups and the combined control groups. It should be emphasized that the differences between the groups were greater for nMT than cMT expression. Furthermore, our observations revealed a stronger nMT expression in the cancer group (81.8%) as compared to benign endometrial hyperplasia (37.5%), what was the reason why the combined control groups were also analyzed. Results obtained by us and Wicherek et al., demonstrate that increased nMT expression is associated with an increasing degree of neoplastic transformation in endometrial tissue. Another interesting finding of our study, which highlights the importance of nMT expression in endometrial cancer, is the existence of a relationship between G and nMT expression. Ioachim and McCluggage obtained similar results analyzing the relationship between G and expression of MT (combined nMT and cMT fractions). The legitimacy of separating MT into two fractions seems more justified if we consider the regulatory pro-proliferative and anti-apoptotic function of nMT, *i.a.* as a transcription factor. Our study revealed a positive correlation between nMT *vs.* Ki-67 expression in the cancer group and a negative correlation between cMT *vs.* Ki-67 expression in the benign hyperplasia group. The above mentioned findings suggest a need for separate analyses of the MT fractions more frequently, as it may be crucial to clarify the role of this protein in endometrial cancer.

Numerous studies concerning EGFR expression show strong expression of this protein in actively dividing cells, *e.g.* cancer cells. Bansal et al., and Niikura et al., described a variable degree of EGFR overexpression in endometrial cancer cells [25, 26], probably due to increased cellular metabolism and active intercellular communication. Based on the functions and cellular localization, EGFR is used as a binding site for anticancer drugs. In our study, we obtained significantly lower proportion of cases with positive EGFR expression in the cancer group in relation to the control subgroups and the combined control groups. Similar results were obtained by Miturski et al., who compared EGFR expression in endometrial cancer and in normal endometrium in the proliferative phase of the menstrual cycle, and by Gershtein et al., who analyzed EGFR expression in benign hyperplasia and in endometrial cancer [27, 28]. The former research team received

stronger EGFR expression in normal tissue, while the latter in benign hyperplastic samples. In view of our results, it would be useful to analyze the expression of other EGFR family members (*i.e.* HER-2, HER-3, HER-4), what could prove the existence of an impaired expression of these proteins.

Our findings are consistent with the results of Nagai et al., who also showed no correlation between EGFR and Ki-67 expression in endometrial cancer [29]. Based on these results and in light of numerous publications on strong association between an increased proliferative index and unfavorable prognosis of endometrial cancer, it seems safe to conclude that intensified cell division and unfavorable cancer prognosis are not directly associated with EGFR expression. Moreover, analysis of MT and EGFR expression revealed only one significant correlation, nMT *vs.* EGFR in the combined control groups. Lack of correlation between EGFR and MT expression in endometrial cancers might indicate an utterly independent role of these proteins in carcinogenesis.

Conclusions

Our study shows that nuclear metallothionein expression remains to be strongly associated with neoplastic transformation of the endometrium and increases with the grade of histological malignancy. Moreover, our results clearly indicate that there is a need for further research on metallothionein expression in tumor cells, especially based on the distinction between nuclear and cytoplasmic localization. These results suggest that cellular localization of the expression carries additional relevant information.

Oświadczenie autorów:

1. Łukasz Jagielski – autor koncepcji i założeń pracy, przygotowanie manuskryptu i piśmiennictwa – autor zgłaszający i odpowiedzialny za manuskrypt.
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4. Radosław Blok – autor założeń pracy, analizy i interpretacji wyników, przygotowanie korekta i akceptacja ostatecznego kształtu manuskryptu.
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