Studies on selected molecular factors in endometrial cancers

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Abstract

Background. Endometrial carcinomas (EC) differ in etiology, clinical course and prognosis.

Objectives. This multi-center study aimed at a closer recognition of molecular factors linked to heterogeneity of EC by evaluating estrogen and progesterone receptors, proteins dependent on MMR genes, proteins linked to poor prognosis and metastases, and mutations in *BRCA1*.

Material and methods. Using sections of paraffin-embedded preparations, in 115 patients with EC type I and 31 with EC type II, expression of ERα, ERβ1, PR, MLH1, and MSH2 proteins, as well as ARID1A, c-MET and BRCA1, was estimated by immunohistochemistry using specific antibodies.

Results. Expression of ERβ1 was augmented in EC type II, in poorly differentiated cancers and with growing clinical advancement. An augmented expression of ERα was noted in well-differentiated EC and at lower clinical stage. An increased expression of PR and decreased of MLH1 were detected in type I EC. The expression of ARID1A and c-MET proteins showed no differences between the types of EC, stages of clinical advancement or grading. In 51.6% patients with type II EC, a loss of BRCA1 expression was disclosed; in this group of cancers a decreased expression of ERα was noted.

Conclusions. An augmented expression of ERβ1 was linked to type II EC. A higher expression of ERα in EC cancers was associated with a lower histopathological grade. A decreased expression of MLH1 protein was estimated in EC type I. Type II EC may be connected to BRCA1 mutation.

Key words: endometrial cancer, BRCA1, estrogen receptors, MMR, ARID1A

Introduction

According to global statistics, endometrial carcinoma (EC) is diagnosed in 4.8% of the female population. In 2012, EC developed in 319,605 women, of which 76,160 cases were fatal. In Europe since 2005 a stable increase has been noted in morbidity and mortality due to EC. In 2012 almost 100,000 women developed EC, which accounts for 6.2% of all morbidities due to malignant tumors in women.^{1,2}

Multi-year observations indicate that ECs vary in etiology, clinical course and prognosis. Since Bokhman's hypothesis was 1st proposed, 2 types of EC have been distinguished.³

Type I is the most frequently diagnosed in around 80% women: mainly endometrioid, it is linked to an unbalanced estrogen stimulation and metabolic syndrome, manifests a slow course and good prognosis. It carries common receptors for estrogens (ER) and progesterone (P), while their expression depends on the degree of clinical advancement and histological grade. Most of the cancers are sporadic; around 3–5% are linked to mutations in the mismatch repair genes (*MMR*): *MLH1*, *MSH2*, *MSH6* and *PMS2*. In this type of EC, molecular tests demonstrated mutations in *PTEN*, *PIK3CA*, *K-RAS* and β -catenin, as well as microsatellite instability (MSI).^{4–9}

Type II is a non-endometrioid cancer that manifests an aggressive biology, encompassing serous, clear-cell and poorly differentiated cancers. Frequent relapses cause an unfavorable course. It is a carrier of *TP53* (p53) and *HER2/neu* mutations. A proportion of the cancers was demonstrated to carry ER hormonal receptors, the effect of which on the clinical course remains controversial.^{10–15}

Uterine serous carcinoma (USC) is thought to represent a unique type of EC, which should be treated as a distinct morbid unit.¹⁶

The results of studies covered by the Human Cancer Genetic Program (543 unselected female endometrial cancers) and focused on MSI demonstrated that, even though MSI was documented in 21.7% of the studied cases, genetic testing for *MMR* (*MLH1*, *MSH2*, *MSH6* and *PMS2*) detected Lynch syndrome in just 1.8% of cases.¹⁷ Another study showed that MSI was more frequent in metastatic than in primary EC, that it appeared late in tumor development and that it might promote progression.¹⁸ Brinton et al., when reporting the results of the Gynecologic Oncology Group (GOG 210 Trial), highlighted the similarity of endometrioid grading 3 (G3) cancers and type II cancers.¹⁹ The authors supported the hypothesis suggesting heterogeneity of EC type II cancers.

As stated above, the mutation of *PIK3CA* is linked to EC type I, although alterations in *PIK3CA* are also present in USC.²⁰ Studies by Takeda et al. showed that mutations in the *ARID1A* suppressor gene induce an altered expression of many genes, including *MLH1* and genes linked to the *PI3K/AKT* signaling pathway, and are therefore associated with both type I and type II EC.²¹ The *PI3K* signaling

pathway also involves the *MET* proto-oncogene and its HGF ligand. Studies by Bishop et al. proved that the expression of c-MET occurred likewise in USC.²²

In recent years USC has been found to be potentially associated with carriership of a mutated *BRCA1* gene. Bruchim et al. subjected women with histologically documented USC to genotyping of 3 main mutations, including *BRCA1* (185delAG and 5382insc) and *BRCA2* (6174delT).²³ They found that over 25% of women with USC carried mutations in *BRCA1/2*. Similar observations were made in English women in 2013; 68% of patients with USC had suffered from breast cancer before being diagnosed with USC. The authors also proposed that at least a subgroup of USC should be recognized as hereditary breast/ovarian cancer, which might carry prophylactic implications (prophylactic adnexectomy) and therapeutic implications (inhibitors of poly adenosine diphosphate ribose polymerase – PARP).²⁴ The heterogeneity of EC, particular of EC type II, requires further molecular studies.

This study aimed to estimate expression manifested by hormonal receptors (ER α , EB β 1, PR), expression of MSH2 and MLH1 proteins involved in the development of certain endometrial cancers belonging to the Lynch syndrome, expression of proteins linked to poor prognosis and metastases (ARID1A, c-MET), and expression of BRCA1 protein in type II EC, which might indicate that a proportion of the cancers are dependent on mutations in *BRCA1* and represents a proportion of breast/ovarian cancer syndrome.

Material and methods

The study had a multi-center, retrospective character. The research material included archival histopathological preparations of endometrial carcinoma, obtained from 162 patients diagnosed and treated due to EC in 2007–2014 in 8 specialized centers treating the diseases from the oncological gynecology branch. The study group consisted of patients treated primary with surgery. Due to insufficient clinical data in 16 patients, further analyses were conducted on 146 patients. The mean age of the patients was 65.1 years.

In the studied group, EC of endometrioid type was diagnosed in 115 patients (78.8%); 31 patients (21.2%) were diagnosed with EC of non-endometrioid type, including 18 patients with serous cancer (12.3%); 11 patients with clear-cell cancer (7.5%) and 2 patients with mucinous cancer (1.4%).

Seventy-four patients (50.7%) were diagnosed at an early stage of clinical advancement (38 patients manifesting grade IA and 36 patients manifesting grade IB; 26% and 24.7%, respectively). Thirty-seven patients (25.4%) were diagnosed at stage II, 22 patients (15%) at stage III and 13 patients (8.9%) at stage IV, according to International Federation of Gynecology and Obstetrics (FIGO) gynecologic cancer staging system. In 38 patients (25%) endometrial

carcinoma demonstrated a high grade of histological differentiation (G1), 59 patients (38.8%) had an intermediate grade (G2), while 55 patients (36.2%) carried a poorly differentiated tumor (G3) (Table 1).

Tissue material in the form of neoplastic endometrium was fixed in 10% buffered formalin, passed according to classical histopathological techniques and embedded in paraffin blocks. Following the evaluation of hematoxylin and eosin-stained (HE-stained) preparations and diagnosis, further studies were conducted on representative preparations. In order to demonstrate the presence of antigens in the tissue material, antibodies were employed specific for: ARID1A (Novus Biological, Littleton, USA NBP1-88932), ER α (Santa Cruz Biotechnology, Santa Cruz, USA sc-8005, clone D-12), ER β 1 (Zytomed Systems, Berlin, Germany MSK042-05, clone PPG5/10), Met (Santa Cruz Biotechnology, Santa Cruz, USA sc-10, clone C-12), MLH1 (Leica NCL-L, Buffalo Grove, USA NCL-L-MLH1, clone ES05), MSH2 (Invitrogen, Carlsbad, USA 33-7900, clone FE11), PgR (Dako, Santa Clara, USA M3569, clone 636), BRCA1 (Abcam, Cambridge, UK ab16780, clone MS110).

The preparations were incubated in a water bath at 96°C in a citrate buffer, pH 6.0, for 50 min. The activity of endogenous peroxidase was blocked using 3% H₂O₂. The preparations were incubated with the antibody at room temperature for 60 min, followed by 10 min rinsing in tris-buffered saline (TBS). The tissue material was incubated with the EnVision system (DakoCytomation, K5007; Dako, Santa Clara, USA) for 30 min. In all preparations, 3,3'-diaminobenzidine (DAB-3.3) was used to visualize the reaction. Subsequently, the preparations were counterstained with Mayer's hematoxylin, then passed through a row of alcohol to xylene and finally closed under a coverslip.

In immunohistochemical tests the negative control involved a reaction with omission of the primary antibody. Using an Olympus BX 43 light microscope and XC 30 digital camera (Olympus, Shinjuku, Tokyo, Japan), 10 photographs were taken of every stained preparation with the immunohistochemical reaction. The photographs were taken at total magnification of $\times 400$.

In the evaluation of staining intensity reflecting expression of ARID 1A, ER α , ER β 1, Met, MLH1, MSH2 and PR proteins, a 4-degree scale was applied:

- 0 – absence of reaction;
- + – reaction obtained in 1–50 immunopositive cells (cell nuclei or cytoplasm);
- ++ – reaction obtained in 50–75 immunopositive cells;
- +++ – reaction obtained in 75–100 immunopositive cells, in every instance seen in 10 visual fields.

Staining intensity ++ and +++ were considered in further analyses as a positive protein expression.

The expression of BRCA1 protein was evaluated in cancer tissue in patients with non-endometrioid cancer. In the cases where the BRCA1/MLH1/MSH2 expression were evaluated in the studied preparations, it was assumed that the presence of the color reaction indicated an absence

Table 1. Characteristics of patients included in the study

FIGO stage	Grading	Number of patients
Endometrioid adenocarcinoma (n = 115)		
IA	G1	14
	G2	15
	G3	4
IB	G1	9
	G2	15
	G3	8
II	G1	10
	G2	11
	G3	6
IIIA	G2	6
	G3	2
IIIB	G2	2
IIIC1	G1	2
	G2	2
IVA	G2	1
IVB	G1	1
	G2	4
	G3	3
Serous adenocarcinoma (n = 18)		
IA	G3	4
IB	G3	3
II	G3	7
IIIC1	G3	4
Clear cell adenocarcinoma (n = 11)		
IA	G3	1
IB	G3	1
II	G3	2
IIIA	G3	1
IIIC1	G3	3
IVA	G3	2
IVB	G3	1
Mucinous adenocarcinoma (n = 2)		
II	G1	1
IVB	G2	1

FIGO – International Federation of Gynecology and Obstetrics; G1 – endometrial carcinoma demonstrating a high grade of histological differentiation; G2 – endometrial carcinoma demonstrating an intermediate grade of histological differentiation; G3 – poorly differentiated endometrial carcinoma.

of mutations in the *BRCA1/MLH1/MSH2* gene while the absence of a color reaction indicated the loss of protein expression, which may be the result of the *BRCA1* gene mutation and *MLH1/MSH2* mutations or hypermethylation of their promoter in the cancer tissue. The immunohistochemical reaction was detected in both cell nuclei and in the cytoplasm with the use of the ER β 1-specific antibody.

Fig. 1. Nuclear expression of ARID1A: A) in cell lining glands of highly mature adenocarcinoma (immunohistochemical reaction ++); B) in solid tissue (with pronounced reaction +++). Magnification $\times 400$

Fig. 2. Nuclear expression of: A) ER α receptor; B) ER β 1 receptor; C) PR receptor in endometrial adenocarcinoma. Magnification $\times 400$

The c-MET protein manifested a cytoplasmic reaction while a nuclear reaction was shown by reactions detecting MLH1, MSH2, BRCA1, PR, ER α and ARID1A (Fig. 1–5).

Statistical calculations were performed with STATISTICA v. 10 software (StatSoft Inc., Tulsa, USA). The Mann-Whitney, the Kruskal-Wallis and Spearman's tests were used. Statistical significance was set at $p < 0.05$.

Results

Histological type

Based on histopathological diagnosis, the patients were divided into 2 groups: patients with 1) endometrioid type and 2) non-endometrioid type of cancer. The latter group included patients with serous cancer, clear-cell cancer and mucinous cancer. In patients with endometrioid type cancer, a decreased expression of both MLH1 protein and ER β 1 was observed ($p = 0.013$ and $p = 0.035$, respectively) (Fig. 6). A reduced expression of PR receptor was detected (intensity of the reaction of 3–2 vs 1–0) among patients with non-endometrioid EC ($p = 0.041$). The reduced immunohistochemical expression of PR receptor was seen

mainly in patients with serous cancer ($p = 0.022$), while intensified immunohistochemical reactions for MLH1 and ER β 1 involved clear-cell cancers ($p = 0.02184$ and $p = 0.00109$). Following the subdivision of non-endometrial cancers to individual subtypes, the subgroup of patients with clear-cell cancers manifested a reduced expression in the immunohistochemical reaction specific for ER α receptors as compared to the expression noted in endometrioid cancers ($p = 0.048$) and a higher expression of immunohistochemical reaction for ER β 1 as compared to expression noted in serous cancers ($p = 0.02658$). No differences were detected in the expression of immunohistochemical reactions specific for the remaining proteins in the studied groups.

Grading

An increase in the expression of ER β 1 receptor was found in parallel to the decrease in histopathological differentiation (G3 vs G1 $p = 0.003$) (Fig. 7). Patients with G1 or G2 endometrial cancer manifested a higher expression of ER α receptors than patients with a G3 cancer (G1 + G2 vs G3) ($p = 0.011$). No differences were detected in the expression of MLH1, MSH2, PR, c-MET and ARID1A dependent on the histopathological grading of the cells.

Fig. 3. A pronounced cytoplasmic reaction with c-MET-specific antibody in a section of endometrial adenocarcinoma (A and B). No such reaction in cell nuclei. Magnification $\times 400$

Fig. 4. A pronounced nuclear reaction with A) MLH1-specific antibody and B) MSH2-specific antibody in a section of endometrial adenocarcinoma. Magnification $\times 400$

Fig. 5. Positive (A) and negative (B) nuclear reaction with BRCA1 antibody in a section of endometrial adenocarcinoma. Magnification $\times 10$

Fig. 6. The values reflect the proportions of endometrial cancer cases manifesting expression of a given protein in cells within the endometrioid and non-endometrioid types (immunohistochemical reaction of +++/+++). * $p < 0.05$

Fig. 7. The values reflect the proportion of endometrial cancer cases manifesting expression of a given protein in cells of a high, moderate or low histopathological grade (immunohistochemical reaction +++/+++). * $p < 0.05$

International Federation of Gynecology and Obstetrics gynecologic cancer staging system

For the analysis, patients with endometrial carcinoma were subdivided depending on the stage of clinical advancement of the disease into early stage of advancement (IA) and the late stage (IB–IV). Women manifesting stage IA showed a statistically significant higher expression of the ERα receptor ($p = 0.04$). Patients with a more advanced disease manifested an augmented expression of the ERβ1 receptor ($p = 0.02$) (Fig. 8). No differences were detected in the expression of MLH1, MSH2, PR, c-MET and ARID1A, which would depend on the stage of clinical advancement manifested by the disease.

Mutation in BRCA1

Patients with non-endometrioid cancer were subjected to an evaluation for the expression of BRCA1 protein in cancer tissue. Among 31 patients with type II EC, 16 proved to have negative expression of this protein (Table 2).

Among the patients with non-endometrioid type of EC lack of BRCA1, expression was correlated with reduced expression of ERα receptor ($p = 0.02$). No other differences

in the expression of MLH1, MSH2, PR, ERβ1, c-MET or ARID1A were detected among women with positive or negative BRCA1 staining (Fig. 9).

Discussion

Many endometrioid adenocarcinomas are thought to carry receptors for estrogens and progesterone. According to Reid-Nicholson et al., as many as 84% of endometrioid cancers (type I) manifest G1 and G2 maturity express ER receptors, as compared to ER expression in 9–54% of non-endometrioid (serous and clear-cell cancers).⁶ The expression of such receptors manifests a correlation exclusively with histological grading, but not with the clinical stage of the disease.

Estrogen receptors may be present in 2 isoforms: ERα and ERβ, which exhibit distinct functions.²⁵ Estrogen receptor β is thought to function as a guardian of the endometrium; its disturbed expression has been described in most endometrial cancers.²⁶ In our patients, the examination of ERβ1 expression demonstrated an increase in non-endometrioid cancer – type II EC (mainly serous carcinoma and clear-cell carcinoma) (Fig. 6) as compared to type I EC: 43.3 vs 21.9, respectively. The expression of ERβ1 increased parallel with histological grading: it was least pronounced in G1 – 9.1, higher in G2 – 26.0 and highest in G3 – 38.5 (Fig. 7). It also increased with the clinical advancement of the cancer: FIGO IA 11.8 vs FIGO IB–IV 31.7. In studies by Chakravarty et al. a decrease was also noted in the expression of ERβ in endometrioid EC cancers, but no differences were detected in the expression as related to grade.²⁷ On the other hand, on the basis of our studies it may be accepted that an increased expression of ERβ1 in type II EC,

Table 2. BRCA1 protein expression status in studied patients with endometrial carcinoma type II

Histopathological type	FIGO stage	Number of patients with negative BRCA1 expression (n = 16)	Number of patients with positive BRCA1 expression
Serous adenocarcinoma	IA	n = 1	n = 3
	IB	n = 1	n = 2
	II	n = 2	n = 5
	IIIC1	n = 3	n = 1
Clear-cell adenocarcinoma	IA	n = 1	n = 0
	IB	n = 1	n = 0
	II	n = 2	n = 0
	IIIA	n = 1	n = 0
	IIIC1	n = 2	n = 1
	IVA	n = 2	n = 0
	IVB	n = 0	n = 1
Mucinous adenocarcinoma	II	n = 0	n = 1
	IVB	n = 0	n = 1

Fig. 8. The values reflect the proportion of endometrial cancer cases manifesting expression of a given protein in cells of early (IA) or more advanced degree (IB–IV) of clinical stage according to FIGO (immunohistochemical reaction +++/++). * $p < 0.05$

Fig. 9. The values reflect the proportion of endometrial cancer cases of non-endometrioid type manifesting expression of a given protein, as related to the expression of BRCA1 protein (immunohistochemical reaction +++/++). * $p < 0.05$

in the presence of poor differentiation (G3) and a higher stage of clinical advancement, was associated with poor prognosis.

Studies by Kreizman-Shefer et al. demonstrated that early endometrioid cancers preserve their expression of ER and PR, while poorly differentiated and clinically advanced cancers manifested an absence of 1 or both receptors.¹² Similarly, in our studies the expression of ERα was higher in well or moderately differentiated cancers than in G3 cancers (48.5 vs 36.5 – Fig. 7), and it was also significantly higher in IA cancers according to FIGO (Fig. 8, FIGO 1A 55.8 vs FIGO IB–IV 34.6). We could not detect ERα expression in non-endometrioid type II cancers, although Sho et al. described it in 21.2% cases of USC, and this was linked to a poor prognosis.¹⁴

In studies by Togami et al., ER and PR expression in USC was associated with a good prognosis.¹³ In our studies the expression of the progesterone receptor PR was significantly more pronounced in type I endometrioid EC (89.8 in EV type I vs 75 in EC type II), which was consistent with the results of the study by Togami et al.¹³ However, we failed to identify differences in PR expression which would depend on histological grading (G), as indicated in the studies by Reid-Nicholson et al. and those by Zhu et al.^{6,28}

Since, as mentioned above, around 3–5% of EC are linked to mutations in DNA-repair genes (*MMR*), we estimated the expression of the 2 main relevant proteins, products of *MSH2* and *MLH1* genes, responsible for 85% cases of Lynch syndrome in all our patients.^{7,9,29} We detected a lower expression of MLH1 protein in endometrioid cancer (type I 51.9 vs type II 77.4), which may indicate that the *MLH1* gene mutation occurred more frequently in cases of type I EC. Berends et al. noted the loss of MLH1 expression among women with EC connected to Lynch syndrome.³⁰

No abnormalities in the expression of ARID1A protein were revealed in our study. Other studies detected a loss of ARID1A expression in around 30% of EEC cancers, in the progression of atypical hyperplasia to cancer as well as in the induction of many genes, including *MLH1*.^{21,31}

Furthermore, the expression of the c-MET protein demonstrated no change in any of the parameters we examined, despite the evident association between c-MET and poor prognosis and metastasizing documented in other studies on EC.^{32–35}

In 16 of our 31 patients (51.6%) with a diagnosis of EC type II, immunohistochemical tests demonstrated an absent expression of BRCA1 protein, indicating a mutation in *BRCA1*. Such incidence was much higher than described by Bruchim et al.²³ In studies by Raffi et al., 50.5% of USC patients were found to develop breast cancer (17.5% before and 33% following diagnosis of USC), which, according to the authors, suggested that the cases represented a proportion of *BRCA* mutation syndrome.²⁴ In our studies, the significantly reduced expression of the estrogen receptor α in this group of women might additionally indicate a relationship between some USC and *BRCA* mutation; it has been postulated that certain BRCA1 proteins inhibit ERα activity.³⁶

Conclusions

An augmented expression of ERβ1 in EC was linked to type II EC. Higher expression of ERα in EC cancers was associated with a lower histopathological grade. A decreased expression of MLH1 protein was estimated in EC type I, which may indicate a mutation in *MLH1* gene in this type of cancer. Type II EC may be connected to *BRCA1* mutation.

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