

IMMUNOEXPRESSION AND CLINICAL SIGNIFICANCE OF THE PTEN AND MLH1 PROTEINS IN ENDOMETRIAL CARCINOMAS

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Carcinogenesis is a multistep process resulting from mutations in genes controlling the cellular growth, differentiation, apoptosis, and genome integrity maintenance. We investigated relationships between the PTEN and MLH1 immunoreactivity in the cancer cells and the histological subtypes of endometrial carcinoma as well as the survival times of the affected women. The PTEN and MLH1 protein immunoreexpression was also examined separately in both clinicopathological groups of endometrial carcinoma. We estimated the practical use of the proteins as diagnostic and predictive markers.

The histoclinical analysis was performed on 104 patients. The follow-up in all the cases was well known. To assess the expression of both proteins in the cancer cells we adopted a semiquantitative immunohistochemical analysis. We proved that the incidence of the PTEN and MLH1 nuclear positive cells was significantly higher in the serous type than in the endometrioid one. We also demonstrated a strong correlation between both cytoplasmic and nuclear PTEN immunoreexpression and the survival times in the entire cohort.

In conclusion, the PTEN and MLH1 immunohistochemical analysis broadens the microscopic diagnosis of the endometrial carcinomas. However, the PTEN and MLH1 antibodies tests cannot determine the recognition of the cancer, and they should not be regarded as independent prognostic factors.

Key words: endometrial carcinoma, PTEN, MMAC1, MLH1, microsatellite instability.

Introduction

Endometrial carcinoma is one of the most common malignant tumours of the female genital tract. Each year, endometrial carcinoma develops in about 142 000 women worldwide, and 42 000 women die from this cancer [1]. Among all the recognised malignant tumours it ranks the 7th (3.9%) as an incidence and 13th (1.7%) as a cause of death [2].

There are two different clinicopathological groups of this cancer: estrogen-related (type I) and non-estrogen-related (type II). The first one develops from the prolonged estrogen stimulation and follows the endometrial hyperplasia. Usually the estrogen-related tumours are of low grade, good prognosis, and predominantly of the endometrioid type. On the contrary, the non-estrogen dependent cancers develop from the atrophic endometrial tissues in older

women. Usually they are of high-grade, poor prognosis with serous or clear cell morphology [3, 4].

Since mid 1990s there has been much progress made in molecular biology. Thanks to it, it turned out that the morphological differences in the clinicopathological groups were mirrored in their molecular genetic profile. While most serous cancers (type II) contain mutations of TP53 and RB genes, endometrioid adenocarcinomas (type I) usually demonstrate the microsatellite instability (MSI), or a specific mutation of the PTEN, K-RAS, and β -catenin genes [5, 6].

The PTEN tumour suppressor gene (phosphatase and tensin homologue deleted on chromosome 10), identified in 1997 and located on chromosome 10 (10q23.3), encodes a 403-amino acid product, which is a dual-specificity phosphatase with both protein and lipid phosphatase activity [7, 8]. The PTEN gene function is to inhibit cellular proliferation, sur-

vival and growth by inactivating the PI 3-kinase-dependent signalling. When the PTEN protein works properly, it acts as part of chemical pathway that makes cells stop dividing and undergo apoptosis when necessary. The PTEN protein inhibits also cell migration, spreading, and focal adhesion formation. There is also some evidence that the protein made by the PTEN gene governs normal vascular development and tumour angiogenesis [9-14].

Research on microsatellite instability has brought on the discovery of the DNA repair genes which are responsible for removing errors that may occur in the DNA during synthesis. The loss of their function renders the DNA susceptible to progressive accumulation of mutations. When the mutations affect proto-oncogenes or tumour suppressor genes, there is a considerable risk of developing cancer. The MLH1 gene (hMLH1, MutL homolog 1) is one of the genes which encode proteins involved in the mismatch repair. Its identification is directly linked with the genesis of the HNPCC syndrome. The MLH1 gene is located on chromosome 3 (3p21.3) and encodes a 756-amino acid product [15-17].

In the present study, we set out to:

- assess the immunoreactivity of the PTEN and MLH1 proteins in endometrial carcinoma,
- find out potential relationships between the proteins and the histological subtypes of endometrial carcinoma,
- find out potential relationships between the PTEN and MLH1 proteins in the cancer tissue and the survival times of the affected women with endometrial carcinoma.

Materials and methods

Patients

The histoclinical analysis was performed on 104 patients with endometrial carcinoma who underwent hysterectomy at the Voivodeship Hospital in Kalisz (Poland) between 1993 and 2004. All the patients came from the same selected region. Their follow-ups were well known until the end of November 2005. Each patient's survival time was estimated in months from the date of diagnosis to the date of death (overall survival).

After the gross examination, the paraffin-embedded tissue blocks and histological slides (HE) were prepared. The histological forms of endometrial carcinoma were as follows: endometrioid adenocarcinoma (involving a variant with squamous differentiation), serous adenocarcinoma, clear cell adenocarcinoma and mucinous adenocarcinoma. All the cases were surgically staged using the FIGO cancer staging system (2009) as IB (tumour invades more than half of the myometrium).

Immunohistochemistry

Paraffin sections were mounted onto SuperFrost slides, deparaffinized, then treated in a microwave oven in a solution of citrate buffer, pH 6.0 for MLH1 and pH 9.0 for PTEN, for 30 min (2×5 min 360 W, 4×5 min 180 W) and transferred to distilled water. Endogenous peroxidase activity was blocked by 0.3% hydrogen peroxide in distilled water for 30 min, and then sections were rinsed with Tris-buffered saline (TBS, DakoCytomation, Denmark) and incubated with monoclonal mouse anti-human: MLH1 (clone G168-15, BD Pharmingen, dilution 1 : 200) and PTEN (clone 6H2.1, Dako Cytomation, dilution 1 : 250). Afterwards EnVision+System-HRP for mouse (DakoCytomation, Denmark) prepared according to the instructions of the manufacturer were used. Visualisation was performed by incubating the sections in a solution of 3,3'-diaminobenzidine (DakoCytomation, Denmark). After washing, the sections were counter-stained with haematoxylin and coverslipped.

For each antibody and for each sample a negative control was processed.

Negative controls were carried out by incubation in the absence of the primary antibody and always yielded negative results.

Statistical methods

The semiquantitative immunohistochemical analysis was done under the light microscope to assess the expression of both proteins in the cancer cells. The intensity of staining was classified separately for the nucleus (PTEN) and the cytoplasm (PTEN and MLH1) and subjectively graded: 1 – no staining, 2 – very weak positive staining, 3 – weak positive staining, 4 – strong positive staining, or 5 – very strong positive staining.

The survival analysis was accomplished for such clinical and histological categories as age, histological subtype of endometrial carcinoma, grade of the cancer, PTEN and MLH1 immunoexpression in the cancer cells. All the analyses were performed with the use of the Statistica 7.0 package. The value of $p < 0.05$ was considered statistically significant. The survival curves were constructed according to the Kaplan-Meier method, and all the differences were tested using the log-rank statistic. The Cox proportional hazards regression model was used to analyse some relationships between the analysed variables and the survival time.

Results

The mean age of the patients was 64.5 ± 10.9 years (range: 41-90). While being operated on, the one year older patients ran 5% higher risk of death from the cancer than the younger women ($p < 0.0067$).

8% of the women died within the first post-operation year. Five-year survival time was 73% (95% CI: 63-83%). After 10 years, 62% (95% CI: 48-76%) of the patients were still alive. The most common histological type of all the endometrial carcinomas turned out to be endometrioid adenocarcinoma (56/54%). The second one was its variant with squamous differentiation (28/27%) and the third – serous adenocarcinoma (18/17%). In some single cases we recognised clear cell adenocarcinoma (1/1%) and mucinous adenocarcinoma (1/1%), which, being statistically insignificant, were not taken into further consideration. The women who had been found to have the endometrioid type of the cancer or its variant with squamous differentiation had more chance to survive than those with serous carcinoma ($p < 0.001$). Five-year survival time was as follows: 80% (95% CI: 68-92%) for endometrioid carcinoma, 81% (95% CI: 65-97%) for endometrioid carcinoma with squamous differentiation, and 26% (95% CI: 4-48%) for serous adenocarcinoma.

Among all the endometrial cancers (104), 74% (77) of the tumours were moderately differentiated (G2 group), 14% (15) – well-differentiated (G1 group), and 12% (12) – poorly differentiated (G3 group). Among all the endometrioid adenocarcinomas the highest rate of death (29%) was recorded in G3, and the lowest (8%) in G1 (22% in G2). The percentages of the patients dying from serous adenocarcinomas were 50%, 73% and 60% for G1, G2 and G3, respectively.

The PTEN and MLH1 immunoreactivity in all the endometrial adenocarcinomas is presented in Table I.

There was not any strong correlation between the nuclear immunoreexpression of the MLH1 proteins and the survival time ($p = 0.0766$). On the contrary, the immunoreactivity of the PTEN protein, both cytoplasmic and nuclear, turned out to be statistically significant ($p < 0.05$). The correlation was as follows: the higher level of the PTEN positive cells, the lower survival chance. The best survival prognosis was for those women who had been found to have the endometrioid type of the cancer and have no or at least very slight immunoreactivity of the PTEN protein in the cytoplasm of the cancer cells. On the other hand, the patients who suffered from serous adenocarcinomas were subject to five times higher risk of death than those with the endometrioid type of the cancer, regardless of the PTEN cytoplasmic immunoreexpression.

The PTEN and MLH1 proteins immunoreactivity was also investigated separately in both clinicopathological groups of endometrial carcinoma. All the endometrioid adenocarcinomas and the endometrioid carcinomas with squamous differentiation (84) were classified as type I, the remaining 18 cases of serous carcinomas were classified as type II. The results are presented in Tables II-IV.

We proved that the incidence of the PTEN and MLH1 nuclear positive cells was significantly higher in the non-endometrioid serous type than in the endometrioid type ($p < 0.05$). The PTEN immunoreexpression in the cancer cell cytoplasm in both estrogen-related and non-estrogen-related cancers was comparable (Table V).

Table I. The intensity of MLH1 and PTEN immunostaining in endometrial adenocarcinomas

INTENSITY OF IMMUNOSTAINING	MLH1 – NUCLEUS		PTEN – NUCLEUS		PTEN – CYTOPLASM	
	N	%	N	%	N	%
1	67	64	39	38	8	8
2	5	5	37	36	15	14
3	12	12	20	19	25	24
4	19	18	7	7	39	38
5	1	1	1	1	17	16

Table II. The nuclear intensity of MLH1 immunostaining in endometrial adenocarcinomas of type I and type II

ENDOMETRIAL CARCINOMA	MLH1 – NUCLEUS 1		MLH1 – NUCLEUS 2		MLH1 – NUCLEUS 3		MLH1 – NUCLEUS 4		MLH1 – NUCLEUS 5		N
	N	%	N	%	N	%	N	%	N	%	
	Type I	59	70	3	4	8	10	14	17	0	
Type II	7	39	2	11	3	17	5	28	1	6	18
n	66	65	5	5	11	11	19	19	1	1	102

Table III. The nuclear intensity of PTEN immunostaining in endometrial adenocarcinomas of type I and type II

Endometrial CARCINOMA	PTEN – NUCLEUS 1		PTEN – NUCLEUS 2		PTEN – NUCLEUS 3		PTEN – NUCLEUS 4		PTEN – NUCLEUS 5		N
	N	%	N	%	N	%	N	%	N	%	
	Type I	35	42	31	37	13	15	4	5	1	
Type II	4	22	5	28	6	33	3	17	0	0	18
n	39	38	36	35	19	19	7	7	1	1	102

Table IV. The cytoplasmic intensity of PTEN immunostaining in endometrial adenocarcinomas of type I and type II

Endometrial CARCINOMA	PTEN – CYTOPLASM 1		PTEN – CYTOPLASM 2		PTEN – CYTOPLASM 3		PTEN – CYTOPLASM 4		PTEN – CYTOPLASM 5		N
	N	%	N	%	N	%	N	%	N	%	
	Type I	7	8	13	15	20	24	30	36	14	
Type II	1	6	1	6	5	28	8	44	3	17	18
n	8	8	14	14	25	25	38	37	17	17	102

Table V. The MLH1 and PTEN expression in endometrial adenocarcinomas of type I and type II

	TYPE I		TYPE II		P-VALUE
	MEAN VALUE \pm SD	MEDIAN (IQR)	MEAN VALUE \pm SD	MEDIAN (IQR)	
MLH1 – nucleus	1.7 \pm 1.2	1.0 (1.0-3.0)	2.5 \pm 1.4	2.5 (1.0-4.0)	0.0363
PTEN – nucleus	1.9 \pm 0.9	2.0 (1.0-2.0)	2.4 \pm 1.0	2.5 (2.0-3.0)	0.0319
PTEN – cytoplasm	3.4 \pm 1.2	4.0 (3.0-4.0)	3.6 \pm 1.0	4.0 (3.0-4.0)	0.4717

Discussion

Endometrial carcinoma, like any other cancer, has a genetic basis and results from sequential acquisition mutations in genes that control the cellular growth, differentiation, apoptosis, and genome integrity maintenance.

The PTEN tumour suppressor, also known as MMAC1 and TEP1, has recently turned out to play an important role in the pathogenesis of various human cancers. The PTEN gene inactivating mutations are present in a significant percentage of endometrial carcinomas (mainly estrogen-related tumours), melanomas, prostate cancers and glioblastomas. The reduced PTEN expression is also found in many other malignant tumours of the lung, liver and breast. The germline PTEN mutations incite several other, very rare disorders like the Cowden syndrome, Bannayan-Riley-Ruvalcaba syndrome, Proteus syndrome, and Proteus-like syndrome. All the disorders caused by the PTEN mutations are called the PTEN hamartoma tumour syndromes, or PHTS [12, 18, 19].

In our research, we adopted a semiquantitative immunohistochemical analysis to assess the expres-

sion of the PTEN and MLH1 proteins in different histological subtypes of endometrial carcinoma as well as in both clinicopathological groups of the cancer. We demonstrated a strong correlation between both cytoplasmic and nuclear immunoreexpression of the PTEN protein and the survival times in all the cohort. Interestingly, it turned out that the higher level of the PTEN positive cells, the lower survival chance. It is at variance with some other researchers' findings. According to Salvesen *et al.*, the loss of the PTEN expression in the endometrial cancer cells is significantly associated with a metastatic disease [20]. Also Depowski *et al.*, who analysed the protein product of the PTEN gene expression in breast cancers, proved that the protein loss was correlated with the cancer-related death, lymph node metastasis, and the loss of the estrogen receptor staining [21].

In the present study, we showed the lack of the PTEN protein nuclear immunoreexpression in 42% of the estrogen-related tumours (endometrioid adenocarcinomas + endometrioid adenocarcinomas with squamous differentiation). In 37% of the cases we classified the staining intensity as very weak. Only in one case we proved a very strong PTEN immunoreexpression. On the other hand, the lack of the protein

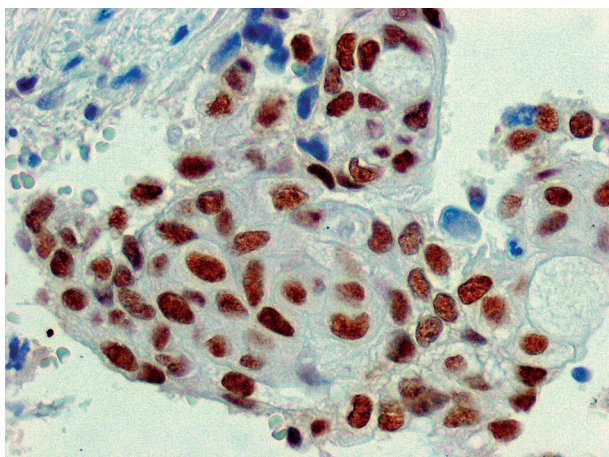


Fig. 1. Very strong nuclear staining for MLH1 in endometrioid adenocarcinoma (variant with squamous differentiation). Original magnification 500×

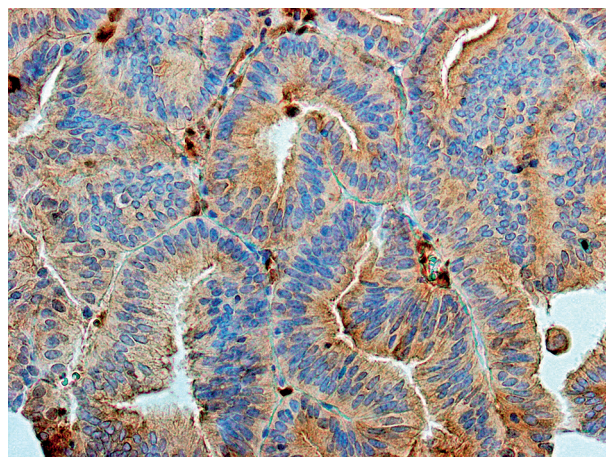


Fig. 2. Very strong cytoplasmic staining for PTEN and negative nuclear expression of PTEN in serous adenocarcinoma. Original magnification 200×

nuclear immunoreactivity in the cancers of type II (serous adenocarcinomas) was detected only in 22% of the cases. The PTEN cytoplasmic immunorepression, comparing to the nuclear one, for both the estrogen-related and non-estrogen-related cancers was not statistically significant. Taking everything into account, we demonstrated that the lack of the PTEN proteins nuclear immunorepression was symptomatic for endometrioid cancers. Our findings are similar to those of some previous research. Nevertheless, the data obtained from some other studies indicate the higher level of the PTEN negative cells in the estrogen-related cancers. For instance, Mutter *et al.* showed the lack or at least very slight immunoreactivity of the PTEN protein in 97% (32/33) of the patients with endometrioid cancers [22]. In our opinion, those differences can be explained due to different interpretations of immunohistochemical stains. Taking it into consideration there is an open question which one is the most reliable. Using an immunohistochemical reaction scale as a percentage of immunopositive cells, when it is not supported by a computer image analyzer, is controversial and can be questioned. One should decide whether to adopt only a quantitative method (the number of immunopositive cells per 1000 or 10 000 cells in research) or a partly subjective semiquantitative method, classifying the intensity of staining in accordance with the methodological assumptions presented in our study. How to standardize various interpretations of immunohistochemical stains remains a completely different question, which is well beyond our research.

Transformation to neoplastic cells can be also triggered by the microsatellite instability (MSI). Microsatellites are short repeated DNA sequences that occur throughout the genome, both within coding and noncoding regions of the genes. Due to their

repetitive sequences, they have an increased susceptibility to mutation during the DNA replication. The DNA repair genes, sometimes called the mutator genes, are those involved in the mismatch repair. The loss of their function can be associated with several cancers including colorectal, endometrial, ovarian, brain and gastric ones [23]. One of these genes – MLH1 was the subject of our research.

Many previous studies have proved that the MSI is involved in the endometrial carcinogenesis, and in particular it is responsible for pathogenesis of type I cancers. According to Modica *et al.* or Hardisson *et al.*, the immunohistochemical analysis of the hMLH1 expression is a useful technique of detecting the MSI in endometrial carcinomas [24, 25]. In our study, we were able to demonstrate the lack of the MLH1 expression in 66 of all the endometrial cancers. 70% of the estrogen-related cancers turned out to be MLH1 negative (the rate for the serous adenocarcinomas was only 39%). So, it means that the incidence of the MLH1 nuclear positive cells was significantly higher in the non-endometrioid serous type than in the endometrioid one.

Although the microsatellite instability (MSI) is commonly known as a prognostic marker in colorectal cancers, its relationship with endometrial cancer prognosis is still controversial for many researchers. Arabi *et al.* proved that there was no statistically significant difference in the survival time between the patients with the MSI tumours and those with microsatellite stable tumours ($p = 0.70$) [26]. Others like Kobayashi *et al.* or Caduff *et al.*, conclude that the MSI phenotype of endometrial carcinomas is associated with a high grade and poor prognosis [27, 28]. Putting a question if there was any correlation between the MLH1 immunoreactivity and the survival times we can state that it was merely statistically significant ($p = 0.0766$).

It is of interest to identify whether the loss of the MLH1 gene function induces the PTEN mutation. Matias-Guid *et al.* proved that from 60% up to 86% of the MSI positive tumours demonstrated also the lack of the PTEN gene function (in the MSI negative endometrial carcinomas the rate was only 24-35%). In their research the PTEN mutations were detected in 2 short coding mononucleotide repeats (A)5 and (A)6 in 4 of 10 (40%) MSI+ endometrial tumours. The data suggest that the PTEN (A)5 and (A)6 mutations may be secondary to deficiencies in the mismatch repair [29].

To sum up our results, we may hypothesize that the immunohistochemical analysis of the protein products of the PTEN and MLH1 genes in endometrial carcinomas broadens the microscopic diagnostics of the tumours. However, the PTEN and MLH1 antibodies test results can neither determine the recognition of the cancer nor the survival prognosis. The most important prognostic factors still are:

- histological type of the cancer,
- patients' age, and
- stage.

At the present state of our knowledge, such biomarkers as the PTEN and MLH1 should be treated as some extra tools to identify estrogen-related and non-estrogen-related endometrial cancers, which lets us recognize which group the patient belongs to.

Conclusions

1. The decreased level of the PTEN and MLH1 immunoeexpression in the cancer cells proves the loss of function of both the PTEN suppressor gene and the MLH1 mutator gen.
2. The lack of the nuclear immunoeexpression of the PTEN and MLH1 proteins in endometrioid cancer comparing to a higher level of the PTEN and MLH1 cells in serous endometrial cancer proves that analysing their immunoreactivity may be a useful and helpful tool to identify estrogen-related cancers.
3. The best survival prognosis: endometrioid type of the cancer and no, or at most east very slight, immunoreactivity of the PTEN protein in the cytoplasm of the cancer cells.
4. Negative prognostic factors: advanced age, serous type of endometrial cancer and the increased level of the PTEN cells in the cancer tissue.

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