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Analysis of polymorphisms / mutations of *PTEN, CDKN2A, TP53* genes and of *hMSH6* gene in endometrial hyperplasia and carcinoma

Analiza polimorfizmów/mutacji genów *PTEN, CDKN2A, TP53* oraz genu *hMSH6* w rozrostach oraz rakach endometrium

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Abstract

Endometrial cancer belongs to the most frequent malignancies in the female genital organs with a growing incidence trend. The molecular changes, which are specific for particular stages of neoplastic transformation or the histopathological type of neoplasm, have not yet been described in any more uniform way. The goal of the undertaken studies was the evaluation of the polymorphisms / mutations of *PTEN, CDKN2A, TP53* suppressor genes and of *hMSH6* gene of incorrectly paired base-pairs in a group of female patients with endometrial hyperplasia and endometrial cancer. The studies involved forty-four (44) female patients: five (5) cases, despite the fact that clinical inclusion criteria had been met, no hyperplastic features were confirmed in a histopathological analysis. Twenty-six (26) patients with histopathologically confirmed endometrial hyperplasia and thirteen (13) patients with diagnosed carcinoma of uterine body mucosa were added into the study. The sequencing method was used for the identification of mutations / polymorphisms in *MSH6, CDKN2A, PTEN* and *TP53* genes. Two changes were identified in *TP53* gene: R175H G>A (CGC>CAC) polymorphism in exon 5 and R213R (CGA>CGG) synonimic change in exon 6 in 15% of the examined patients (2/13 cases of endometrial cancer). Regarding DNA sequence of exon 3 in MSH6 gene, the following polymorphism was found in 29% of the patients: D180D (GAT>GAC). None of the patients demonstrated any changes, either in DNA sequence of *CDKN2A* gene or in exons 2, 5, 7 and 8 of the *PTEN* gene.

Key words: endometrial hyperplasia, endometrial cancer, *PTEN, CDKN2A, TP53, hMSH6.*

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Streszczenie

Rak endometrium należy do najczęstszych nowotworów złośliwych żeńskich narządów płciowych. Rejestruje się stały wzrost liczby zachorowań. Dotychczas nie zostały jednoznacznie opisane zmiany molekularne, specyficzne dla poszczególnych etapów transformacji nowotworowej lub typu histopatologicznego nowotworu. Celem podjętych badań była analiza polimorfizmów/mutacji genów supresorowych *PTEN, CDKN2A, TP53* oraz genu naprawy błędnie sparowanych zasad hMSH6 w grupie pacjentek z rozrostem endometrium oraz rakiem endometrium. Badaniami objęto 44 pacjentki: w 5 przypadkach, pomimo spełnienia klinicznych kryteriów włączenia, histopatologicznie nie potwierdzono cech rozrostu, do dalszych badań włączono 26 pacjentek z histopatologicznie potwierdzonym rozrostem endometrium oraz 13 pacjentek ze zdiagnozowanym rakiem błony śluzowej trzonu macicy. Do identyfikacji mutacji/polimorfizmów w genach MSH6, *CDKN2A, PTEN* i *TP53* wykorzystano metodę sekwencjonowania. W genie *TP53* wykryliśmy dwie zmiany: polimorfizm R175H G>A (CGC>CAC) w egzonie 5 oraz zmianę synonimiczną R213R (CGA>CGG) w egzonie 6 u 15% badanych (2/13 przypadków raka endometrium). W sekwencji DNA egzonu 3 genu MSH6 u 29% pacjentek został wykryty polimorfizm: D180D (GAT>GAC). U żadnej z pacjentek nie została wykryta zmiana w sekwencji DNA genu *CDKN2A* oraz w egzonach 2; 5; 7 i 8 genu *PTEN*.

Słowa kluczowe: rozrost endometrium, rak endometrium, *PTEN, CDKN2A, TP53, hMSH6*

Introduction

Endometrial carcinoma is one of the most frequent malignancies in female genital organs observed in the Western countries with high living standards. Its annual incidence is estimated at 15-20 new cases per 100 thousand of women [1, 2].

The identification of the genetic origins and basis of neoplastic disease becomes a significant element of the diagnostic and classification apparatus. Histopathology results are more and more often supplemented with molecular diagnostics, just as it is in the cases of breast cancer or hyperplastic blood diseases.

Molecular studies on the pathogenesis of endometrial cancer have unveiled a number of genetic changes, both hereditary and somatic, the accumulation of which is observed in the course of neoplastic transformation. This type of cancer develops via two mechanisms of carcinogenesis. These two ways are reflected in the endometrial cancer division into the following two types: endometrioid (type I) and non-endometrioid (type II) [3-6]. Type I cancers are associated with mutations of *KRAS2* oncogene, mutations of the *PTEN* suppressor gene and β-catenin, defects in the repair system of incorrectly paired base-pairs in DNA (MMR, DNA mismatch repair) and with diploidal karyotype; whereas type II cancers are associated with mutations of *TP53* and *CDKN2A* gene and with the overexpression of *ERBB-2* (HER-2/neu) gene [7]. The criteria of the above mentioned division are not unequivocal as there are tumours demonstrating molecular features of both type I and type II cancers.

Material and methods

The study involved forty-four (44) female patients with perimenopausal bleeding episodes, resistant to pharmacological therapy. In five (5) cases, histopathology did not indicate any hyperplastic features, while in twenty-six (26) cases endometrial hyperplasia was confirmed in the histopathological evaluation of collected material. No atypy features were identified, either in six (6) patients with simple hyperplasia or in eleven (11) patients with microscopically complex hyperplasia. Cellular atypy was observed in two (2) patients with simple hyperplasia and in seven (7) patients with complex hyperplasia. Endometrial cancer was diagnosed in thirteen (13) patients, including seven (7) cases with G1 degree of histological malignancy, while G2 degree was diagnosed in six (6) cases. All the cases of cancers were histologically diagnosed as endometrioid adenocarcinoma.

At the time of surgical intervention the age of the patients varied from 39 to 87 years. The patients excluded from the study were those with concomitant malignancy, either diagnosed or in history.

The population of the control group included fifty-four (54) female patients at the age from 20 to 80 , while the reference group consisted of female subjects with no cancer in history. The research project obtained a positive opinion

of the Committee of Bioethics at the Medical University of Lodz, Approval No. RNN/80/10/KE. Each time, the doctor informed a given patient about the study goals and received a voluntary consent to participate in the research project.

In hyperplasia cases, the material for the analysis was collected via negative pressure transcervical aspiration and immediately placed either in Bouin's fluid (for histopathological studies) or in liquid nitrogen (-196°C) (for molecular studies). Peripheral venous blood was another material for molecular evaluations.

In each case of malignant changes the collected material consisted of the samples of peripheral blood, tumour fragments and specimens of the healthy myometrial tissue. The collected material was secured as specified above. Evaluations were carried out by two experienced pathologists from two independent diagnostic centres. Molecular studies were performed in all the types of clinical specimens for each patient.

DNA was isolated by the column method, using a Sherlock AX kit of the A&A Biotechnology Company. The quality of obtained DNA was assessed by a NanoDrop ND-1000 spectrophotometer.

The Real-time HRM technique was used for genomic DNA replication, which enabled the identification of different variants of the amplified genes and the sequencing method was applied to identify the found polymorphisms/mutations. For the amplification of genomic DNA Precision Melt Supermix of the BioRad Company (www.bio-rad.com) was used. The set included: iTaq hot-STRT DNA polymerase, dNTPs, $MgCl_2$ buffer ions, EvaGreen dye and a mixture of amplifiers and stabilizers optimized for the reaction of HRM. We used 1-50 ng of genomic DNA in PCR reaction. The reaction mixture was prepared according to the manufacturer's instructions. All primers used in the PCR reactions are shown in the table number I below.

Table I. The sequences of primers, melting temperatures, and the size of PCR products.

Gen	Starter F 5'→3'	Starter R 5'→3'	Temp. °C	bp
CDKN2A-exon 1	GAA GAA AGA GGA GGG GCT G	GCG CTA CCT GAT TCC AAT TC	50.3	340
CDKN2A-exon 2	GAA AAT TGG AAA CTG GAA GC	TCT GAG CTT TGG AAG CTC T	51.8	508
CDKN2A-exon 3	CCG GTA GGG ACG GCA AGA GA	CTG TAG GAC CCT CGG TGA CTG	57.9	169
MSH6 exon 1p1	TCC GTC CGA CAG AAC GGT TG	TTC GCG TGA GGC CCT GGC CGA	58	208
MSH6 exon 1p2	CGC TGA GTG ATG CCA ACA AG	CAA CCC CCT GTG CGA GCC TC	58	355
MSH6 exon 2	AAC TAA GTT ATG TAT TTC CT	CCT GTC TGT CTG TTT CTC TC	50	334
MSH6 exon 3	CTG GTC TTG AAC TGC T	CCC CTT TCT TCC CCC ATC	58	289
MSH6 exon 4p3	CGT TAG TGG AGG TGG TGA TG	ATG AAT ACC AGC CCC AGT TC	55	353
MSH6 exon 4p5	CCT CTG AGA ACT ACA GTA AG	CCA AAA CTG GGA GCC GGG TA	52	333
MSH6 exon 4p7	CCT CAA AAA ATG CCT TAT TG	AGC CAT TGC TTT AGG AGC CG	52	304
MSH6 exon 4p8	ACT TGC CAT ACT CCT TTT GG	TCC AGA GCA GAA AGA AAA TC	50	339
MSH6 exon 4p10	GAA AAG GCT CGA AAG ACT GG	TCG TTT ACA GCC CTT CTT GG	52	301
MSH6 exon 6	GTT TAT GAA ACT GTT ACT ACC	GCA AAT ATC TTT TAT CAC AT	52	282
MSH6 exon 9	TTT TGA GAG GGC ACT TCT GT	CCC CTT TTA CTG TTT CTT TG	52	365
TP53 exon 5	TGT GCC CTG ACT TTC AAC TC	AAC CAG CCC TGT CGT CTC TC	58	263
TP53 exon 6	TGA TTC CTC ACT GAT TGC TC	ACC CCA GTT GCA AAC CAG AC	55	157
TP53 exon 7	AAG GCG GAC TGG CCT CAT CT	CAG TGT GCA GGG TGG CAA GT	64	181
TP53 exon 8	GGA CCT GAT TTC CTT ACT GC	GAG GCA TAA CTG CAC CCT TG	58	241
PTEN exon 2	TTT CAG ATA TTT CTT TCC TTA	AAC AAG AAT ATA AAA CAT CAA	40.7	170
PTEN exon 5p1	ACC TGT TAA GTT TGT ATG CAA C	CTT TCC AGC TTT ACA GTG AA	47.7	234

Table I. The sequences of primers, melting temperatures, and the size of PCR products (contiunued).

Gen	Starter F 5'→3'	Starter R 5'→3'	Temp. °C	bp
PTEN exon 5p2	GCT AAG TGA AGA TGA CAA TCA	TCC AGG AAG AGG AAA GGA AA	48.5	201
PTEN exon 7	TGA CAG TTT GAC AGT TAA AGG	GGA TAT TTC TCC CAA TGA AAG	48.5	264
PTEN exon 8p1	TTA AAT ATG TCA TTT CAT TTC TTT T	TTG CTT TGT CAA GAT CAT T	55	233
PTEN exon 8p2	GTG CAG ATA ATG ACA AGG AAT A	TCA TGT TAC TGC TAC GTA AAC	54	263

PCR was performed in 40 cycles, initial denaturation in 95°C for 120 seconds, annealing 45-58°C for 30 seconds, depending on the primer pairs used, extension 72°C for 30 seconds, and a final extension at 72°C for 10 minutes. The HRM analysis was preceded by the formation of hetero-duplexes: 95°C for 30 seconds, then 60°C for 10 seconds. The range of the melting temperature was 65-95^{o}C, the temperature increased by 0.1°C every 10 seconds. The reaction was stopped at 4°C.

The sequencing was carried out with a 3730 DNA Analyzer of the Applied Biosystems Company at the Source Bioscience Sequencing.

Results

Each of the PCR product was subjected to the melting process. After HRM reaction the samples were divided into different groups - genotypes. A representative number of samples from each group was sent for sequencing to detect changes in the DNA sequence. The normalized melting curves of HRM reaction for the exon 3 in *MSH6* gene and the exon 5 in *TP53* gene respectively show figure 1 and 2.

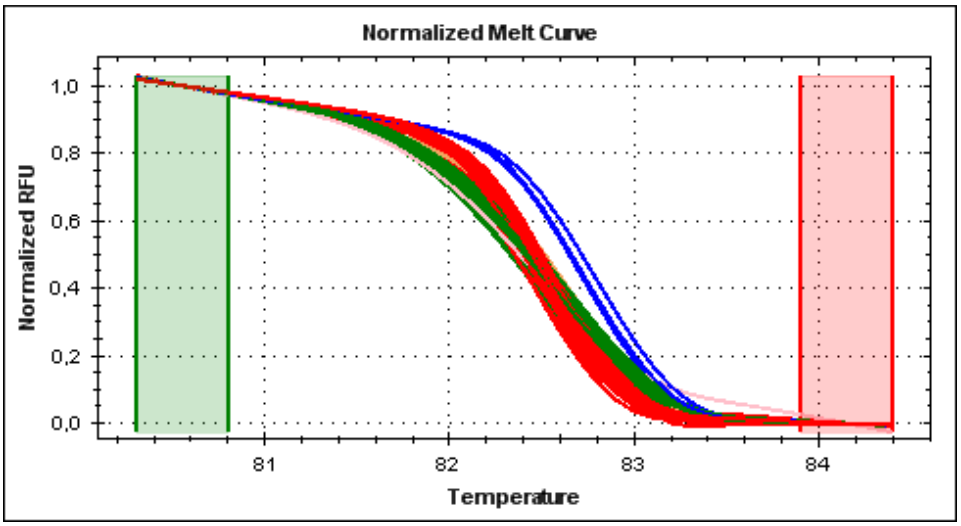

Fig. 1. Normalized melting curve for exon 3 in *MSH6* gene

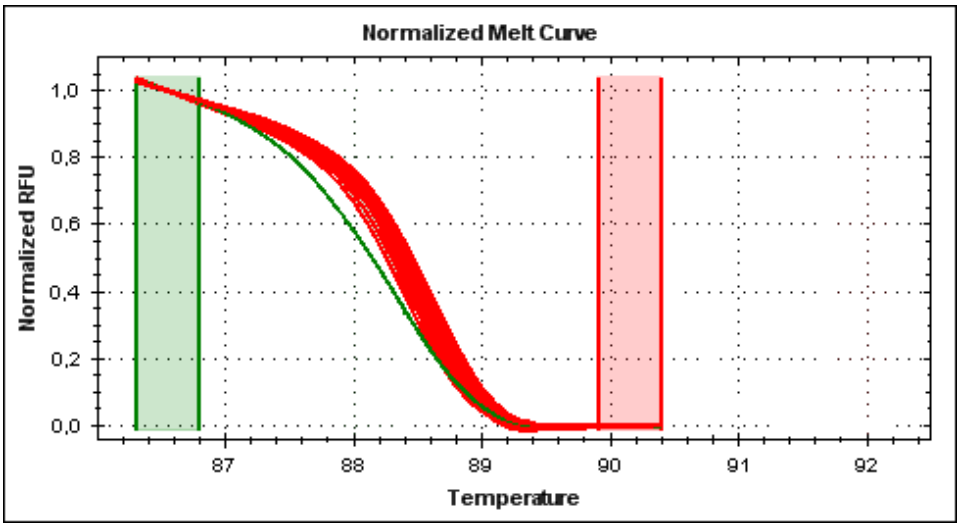

Fig. 2. Normalized melting curve for exon 5 in *TP53* gene

In none of the patients changes in DNA sequence of the *CDKN2A* gene or in exons 2, 5, 7 and 8 of the *PTEN* gene were detected.

In DNA sequence of exon 3 in *MSH6* gene of thirteen (13) (29.5%) patients, D180D (GAT>GAC) polymorphism was found. It is a synonymic change, not exerting any changes in the encoded amino acid, while both GAT and GAC encode tyrosine. The polymorphism of heterozygotic character was identified in ten (10) patients, while the polymorphism of homozygotic character was found in three (3) patients. That change was detected in three types of clinical material: blood, a tumour fragment and in the fragment of the macroscopically unchanged myometrial tissue. See Figures No. 3 and 4 below for the histograms of sequencing reaction, depicting the discussed change.

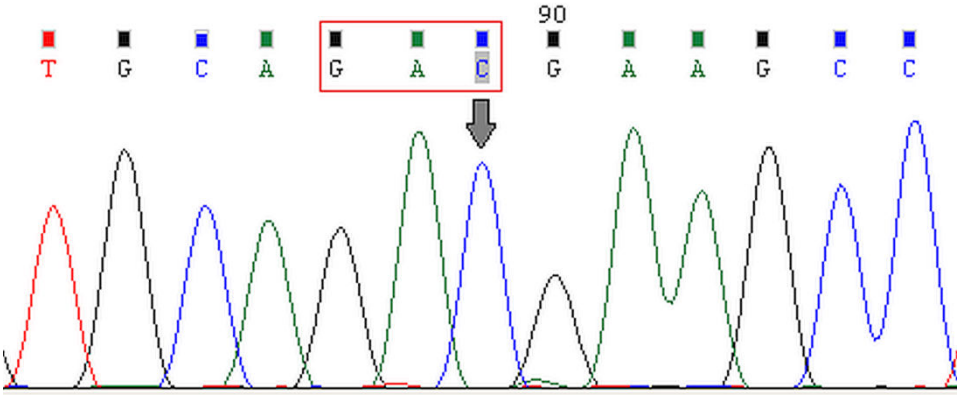

Fig. 3. Histogram of exon 3 sequencing in *MSH6* gene: D180D (GAT>GAC) homozygotic polymorphism.

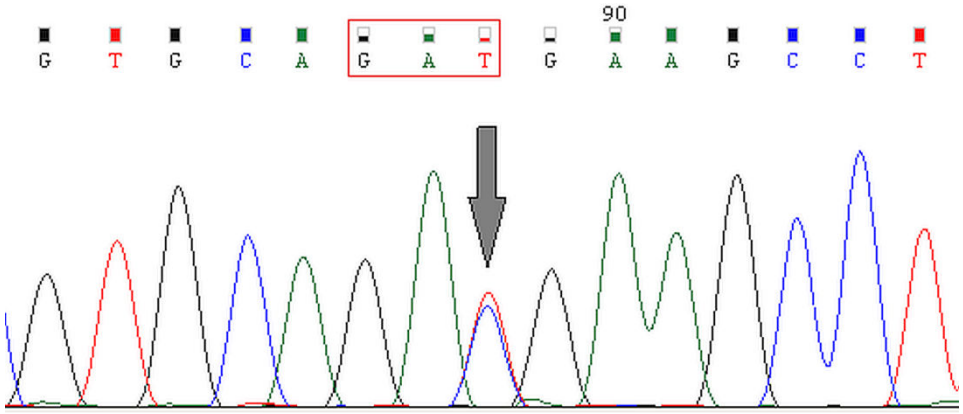

Fig. 4. Histogram of exon 3 sequencing in *MSH6* gene: D180D (GAT>GAC) heterozygotic polymorphism.

In none of the patients changes in DNA sequence in exons 1, 6 and 9 or in the studied parts of exon 4 of MSH6 gene were detected.

In order to evaluate the usefulness of D180D polymorphism in exon 3 of *MSH6* gene as a diagnostic marker, DNA samples from fifty-four (54) patients of the control group were submitted to HRM (high-resolution melting) analysis. D180D polymorphism was not identified in any patient from the control group.

In one (1) patient (2.3%) the following mutation was found in DNA sequence of exon 5 in *TP53* gene: R175H G>A (CGC>CAC). There was a change in the encoded amino acid, where arginine was replaced by histidine. That change was detected in three material types: blood, a tumour fragment and in the fragment of macroscopically unchanged myometrial tissue. See Fig. 5 below for the histogram of sequencing reaction, depicting the described change.

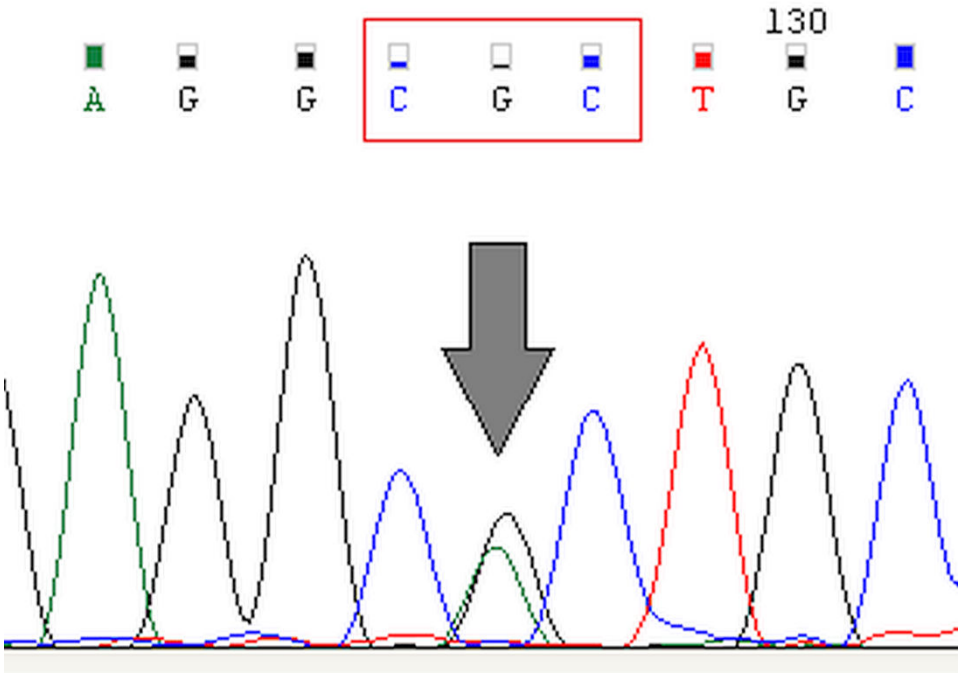

Fig. 5. Histogram of exon 5 sequencing in *TP53* gene: R175H G>A (CGC>CAC) mutation.

The following change was identified in DNA sequence of exon 6 in TP53 gene of one (1) patient (2.3%): R213R (CGA>CGG). It was a synonimic change, not exerting any changes in the encoded amino acid, where both CGA and CGG encodes arginine. That change was found in three material types. See Fig. 6 for the histogram of sequencing reaction depicting the described change. No DNA sequence changes were found in exons 7 and 8 of *TP53* gene in any of the patients.

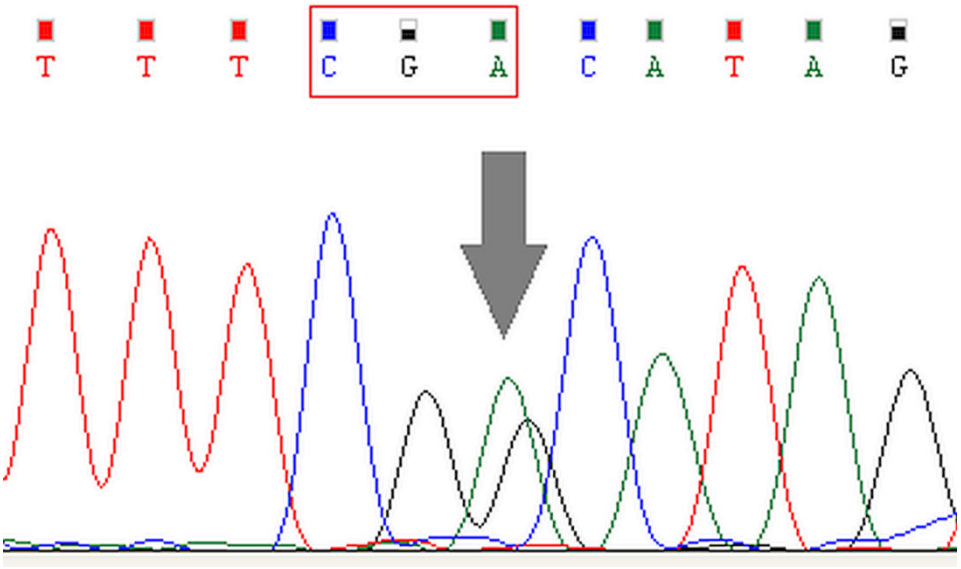

Fig. 6. Histogram of exon 6 sequencing in TP53 gene: R213R (CGA>CGG) change

Discussion

Changes which have been discovered in *CDKN2, PTEN, TP53* and *MSH6* genes may potentially have some relationship with the neoplastic transformation in the endometrium. These genes are mainly associated with DNA repair processes and with the process of apoptosis, thus the processes of key importance in carcinogenesis – also in the cases of endometrial cancer.

Regarding the endometrial cancer, the most frequently observed change in suppressor genes is the mutation in *PTEN* gene, being diagnosed in up to 55% of the neoplasms, more often in endometrial cancers with an accompanying microsatellite instability [6, 8]. Mutations in PTEN gene are also observed in hyperplasia of the uterine mucous membrane [9], thus a changed protein expression could be used as a diagnostic marker of precursor changes [10]. The evaluation of *PTENE* gene mutations may also be useful in the differential diagnostics of primary and metastatic changes of endometrial neoplasms [11]. In the studies by Bussaglia et al., changes in *PTEN* gene were identified in 44% of endometrial tumours. [12]. In turn, Risinger et al. observed mutations

in that gene in 32% of cases [13]. Analysing changes in PTEN gene in hyperplasia, Maxwell et al. found changes in 19% of atypical hyperplasia and in 21% of endometrial hyperplasia without atypy features [13].

Our results of DNA sequence analysis from *PTEN* gene were different from the data, obtained by other authors as no mutations/polymorphisms were found in that gene. The lack of mutations/polymorphisms in the selected sequences of PTEN gene does not, however, exclude their possible occurrence in other regions of the gene, which were not studied. Still the lack of changes in those exons in which molecular events have most frequently been described by other authors may speak in favour of the possible existence of other mechanisms of endometrial hyperplasias and carcinomas which may simply by-pass *PTEN* gene.

The results of sequence analysis of CDKN2A gene in the presented study did not reveal any mutations or polymorphisms. Such a result may be explained by the examined patients being qualified to type one (endometrioid) cancers, in which changes in *CDKN2A* gene are rarely observed.

In endometrial cancers of type II changes in *TP53* gene are observed in approximately 90% of cases, while it is only 17% in endometrioid cancers [14, 15]. These data reflect the fact that in type II cancers mutations in *TP53* gene are early episodes and later events in carcinomas of type I [16-18].

The results of the analysis in our study, targeting DNA sequences in *TP53* gene, demonstrated changes in 15% (2/13) of the patients with diagnosed endometrial cancer. However, we did not find any changes in the studied sequences of *TP53* gene in the patients with identified endometrial hyperplasia. The diagnosed R175H G>A (CGC>CAC) mutation was identified in exon 5 of a patient with diagnosed endometrial cancer with GII malignancy level, whereas R213R (CGA>CGG) change was found in exon 6 of a patient with endometrial cancer with GI malignancy degree. The presented data confirm the fact that changes in TP53 gene are a rare molecular event in type I endometrial cancers, neither being met in the early stage of carcinogenesis of endometrial cancers, that is at the stage of hyperplasia.

R213R change of *TP53* gene is a synonimic change, thus it may be assumed that it either has no relationship with the development process of endometrial cancer or has no influence on the course of the disease. R175H mutation in exon

5 of *TP53* gene may be associated with the increased resistance of endometrial tumours to the therapy and the neoplasms which manifest this change, may be characteristic of a more aggressive course. The interpretation of the results from the experiment by Tsang et al. explains the probable mechanism underlying the decreased response to chemo- and radiotherapy in subjects with R175H mutation in exon 5 of TP53 gene. The cells of the osteosarcoma with R175H mutation in exon 5 of *TP53* gene were not susceptible to cytostatic-induced apoptosis. [19].

A separate group of genes whose damage may predispose to cancer formation constitute DNA repair (mutator) genes. The goal of the undertaken study was, among others, to check whether selected sites in *hMSH6* gene may be useful in prognosing the risk of endometrial cancer progression. The observed accumulation of errors in microsatellite sequences results from the damages of the repair systems, in particular, the system of postreplication repairs, such as, for example, the system of repair of incorrectly paired base-pairs. The microsatellite instability in neoplasms of type I occurs in 20-45% of the cases and is most often identified in type II cancers (9-11%) [20]. In our study, D180D (GAT>GAC) synonimic polymorphism in exon 3 of *MSH6* gene was found in 23% (3/13) of the patients with histopathologically confirmed endometrial cancer. Referring to the patients with diagnosed hyperplasia the change occurred in 34% (9/26) of the examined patients. Analysing the presence of the polymorphism in the context of atypical hyperplasia, that is a change, assumed to be a direct stage, preceding the endometrial cancer formation, it occurred in 33% (3/9) of the patients with hyperplasia with the features of cellular atypy. Our results confirm the thesis that changes in mutator genes are frequent molecular episodes in type I endometrial cancers and occur already in the early stages of carcinogenesis of this neoplasm. Hannemann et al., Samarnthai et al. and Lax et al. described an identical relationship, concerning the occurrence of defects in DNA repair genes in precursor changes of endometrial carcinoma [20-22].

The incidence rate of the identified changes, estimated at 15% for *TP53* gene and at 29.5% for *hMSH6* gene, allows for a careful speculation on the role of changes in those genes as potential risk factors enabling the differentiation of cases with the threat of endometrial cancer formation. A key fact which speaks

in favour of the diagnostic role of changes in *hMSH6* gene is their occurrence at the early stage of carcinogenesis – at the stage of endometrial hyperplasia. Moreover, taking into consideration the obtained results, one may assume that changes in mutator genes occur earlier, already at the stage of hyperplasia, when compared to the changes in suppressor gene *TP53* which manifest their presence only at the stage of endometrial cancer. The data presented over here have also confirmed the retrospective relevance and suitability of histopathological diagnoses in the examined cases qualifying them to type 1 neoplasms. One should, however, remember that the definition of an unequivocal algorithm for the molecular events which lead to the transformation of a normal cell into a neoplastic cell is now rather premature to speak about. The cause(s) should be sought for in the multi-factor differentiation of the pathways which lead to endometrial cancer development on one hand, and in the concomitance of other mechanisms such as chronic infection with oncogenic viruses or the role of hormonal factors on the other.

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