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DNA methylation and the activity pattern of DNA methyltransferase (DNMT1) in endometrial carcinoma

Metylacja DNA i aktywność DNA metylotransferazy (DNMT1) w raku błony śluzowej macicy

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Abstract

Introduction: *Endometrial cancer is the sixth most common malignancy among the female population worldwide, and the most frequently recognized tumor of the female genital tract. DNA methylation plays an important role in the epigenetic regulation of gene expression during cell development and disease status. The complex of events in the DNA methylation pattern greatly depends on the catalytic role of DNA-specific enzymes called methyltransferases.*

Objectives: *The aim of our study was to compare DNA methylation and DNMT1 activity pattern in endometrial cancer tissues collected from three distinct areas of the uterine cavity, and to evaluate the prognostic value of the enzymatic role of DNMT1.*

Material and methods: *The study group consisted of 37 women (28 with the diagnosis of endometrial adenocarcinoma and 9 with uterine fibroids) who underwent hysterectomy at the II Department of Gynecology, Medical University of Lublin, between 2008 and 2009. Tissue specimens were obtained for the purpose of the study from three distinct uterine sites (84 cancerous tissue specimens and 27 healthy endometrium samples in total). A standardized protocol with the use of commercial kits (Epigentek, USA) was used for all of the collected samples. U Mann Whitney and W Shapiro-Wilk tests were used to compare the results.*

Results: *DNA methylation levels as well as DNMT1 activity levels were significantly lower (3.83 vs. 7.65 OD/h/mg; p=0.0022) in the endometrial adenocarcinoma tissues collected from the uterine cavity as compared to healthy endometrial tissues.*

Conclusions: *Global DNA hypomethylation and significantly lower DNMT1 activity observed in the endometrial adenocarcinoma samples in comparison to healthy endometrial tissue can be putative molecular markers of carcinogenesis. Further studies are needed to confirm this hypothesis.*

Key words: **DNA methylation** / **endometrial cancer** / **enzymatic activity** / / **methyltransferase DNMT1** /

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Streszczenie

Cel pracy: *Rak błony śluzowej macicy jest obecnie szóstym nowotworem złośliwym pod względem zachorowalności na świecie w populacji żeńskiej i najczęściej diagnozowanym guzem dróg rodnych. Metylacja DNA odgrywa znaczącą rolę w regulacji epigenetycznej ekspresji genów w trakcie rozwoju komórkowego i chorobie. Szereg zdarzeń w procesie metylacji DNA znacząco zależy od katalitycznej roli enzymów zwanych metylotransferazami. Celem naszej pracy było porównanie globalnej metylacji DNA i aktywności metylotransferazy DNMT1 w tkankach raka błony śluzowej macicy pobranych z trzech różnych okolic jamy macicy i sprawdzenie czy DNMT1 może mieć wartość prognostyczną.*

Materiał i metody: *Grupę badaną stanowiło 37 pacjentek poddanych histerektomii II Klinice Ginekologii UM w Lublinie pomiędzy rokiem 2008 a 2009 (28 pacjentek ze zdiagnozowanym gruczolakorakiem błony śluzowej macicy oraz 9 pacjentek z mięśniakami macicy). Próbki tkankowe pobierane były z trzech różnych okolic jamy macicy (łącznie 84 próbki tkankowe nowotworowe oraz 27 próbek prawidłowego endometrium). Wszystkie zebrane próbki były opracowywane zgodnie z wystandaryzowanym protokołem z użyciem komercyjnych zestawów diagnostycznych (Epigentek, USA). Zastosowano test U-Mann'a Whitneya oraz W Shapiro Wilka do porównania uzyskanych wyników.*

Wyniki: *Poziom globalnej metylacji DNA jak również aktywność enzymatyczna DNMT1 (3,83 vs. 7,65 OD/h/mg, p=0,0022) były istotnie statystycznie niższe w tkankach gruczolakoraka.*

Wnioski: *Globalna hipometylacja DNA oraz istotnie statystycznie niższa aktywność DNMT1 potwierdzona w tkankach gruczolakoraka błony śluzowej macicy w porównaniu ze zdrową tkanką endometrium może stanowić jeden z wielu molekularnych markerów kancerogenezy w raku endometrium. Jednak dalsze badania są konieczne by potwierdzić tę hipotezę.*

Słowa kluczowe: **rak endometrium** / **aktywność enzymatyczna** / **metylacja DNA** / / **metylotransferaza DNMT1** /

Introduction

Endometrial cancer

Endometrial cancer is the sixth most common malignancy among the female population worldwide [1], and the most frequently recognized tumor of the female genital tract [2], with steadily raising morbidity rates [3]. Most of the diagnosed uterine tumors are adenocarcinomas, which are believed to be estrogen-dependent and tend to be lower grade and have fewer recurrences, as well as better survival outcome than other known types of endometrial cancers [4]. Endometrioid endometrial carcinoma (EEC), or type I cancer, accounts for ~75 % of the diagnosed cases. EEC is frequently associated with the general health condition, e.g. nulliparity, early age at menarche, late age at menopause, arterial hypertension, diabetes mellitus, polycystic ovary syndrome, and obesity [5, 6]. EEC is often developed in the background of atypical complex hyperplasia and is characterized by mutations in suppressor gene phosphatase and tensin homologue (*PTEN*) and oncogene *K-ras* and defects in DNA mismatch repair manifested by microsatellite instability (MSI) [7]. It has been also confirmed by Bocker that approximately 90% of EEC cases appeared to be sporadic tumors, while the remaining 10% are recognized as hereditary, often associated with Lynch syndrome, familial syndrome associated with higher risk of colorectal cancer. Most endometrioid endometrial cancers are early diagnosed low grade G1or G2 tumors, which are curable and, with appropriate surgical intervention, have a favorable prognosis. However, the prognosis is significantly less favorable for high-grade (G3) stage [8]. Serous endometrial cancer type II arises from atrophic endometrium, especially in older women. It is poorly differentiated in comparison with EEC and has worse prognosis due to early spread.

DNA methylation and DNA methyltransferase-1 (DNMT1)

Methylation at the 5'carbon position of the pyrimidine ring of deoxycytidine located within CpG dinucleotides represents major epigenetic modification of the human genome [9]. Recent cancer research states that carcinogenesis develops by accumulation of specific genetic events like inactivation of tumor suppressor genes and activation of the oncogene over time [10]. Promoter methylation is an epigenetic process which plays an important role in the development of healthy as well as cancerous cells. In normal cells, it contributes to different epigenetic alternations: chromatin inactivation, tissue specific expression, genetic imprinting, X-chromosome inactivation, or silencing of the transposable elements [10]. In 2008, Esteller proved that CpG island methylation pattern may be gene- and cancer type-specific [11].

During mammalian cellular development, DNA methylation patterns need to be established, maintained and removed. The process of DNA methylation in normal somatic cells in vertebrates is known to be enzymatically controlled by methyltransferases (DNMTs). In mammals, three types of DNMTs have been identified so far: DNMT1, DNMT2, DNMT3 (A,B,L forms). DNMT1, DNMT3A and DNMT3B play pivotal roles in human carcinogenesis by methylating CpG islands in a DNA strand [12, 13, 14]. Hsu et al., described two isoforms of DNMT1 – A and B, in which mRNA expression for DNMT1B is ~30-60% lower in comparison to DNMT1A and is tissue-specific. In addition, DNMT1 appears to be linked with DNA damage repair pathways by preventing mutagenic events. Moreover, inactivation of specific genes, including tumor suppressor genes, by aberrant methylation of the CpG islands has been documented in several

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cancers, including uterine adenocarcinoma [15]. DNMT1 was discovered in 1988 by Bestor as the catalytic enzyme which has a preference for hemimethylated DNA, rather than the unmethylated DNA strand, and is responsible for the symmetrical methylation of nascent DNA strands during DNA replication [16]. Therefore, during DNA replication, DNMT1 can recognize the normally methylated CpG islands in the parent strand and catalyze cytosine methylation in the corresponding CpG site in the daughter strand [17]. In human cancer, an increase in DNMT1 activity is commonly associated with malignancy progression and upregulation of the *DNMT1* gene expression has been described in several cancers tissues, e.g. lung, colon, leukemia, breast, ovarian, and endometrial [18, 19, 20, 21]. However, in a recent report, Espada et al., demonstrated that human DNMT1 can have biological functions which are independent of its DNA 5-cytosine methyltransferase-1 activity [22].

Objectives

The aim of the study was to compare DNA methylation and DNMT1 activity patterns in endometrial cancer tissues collected from three areas of the uterine cavity in order to check 5-cytosine methyltransferase-1 activity pattern in uterine adenocarcinoma tissue, and evaluate the prognostic value of the enzymatic role of DNMT1. Lack of properly designed, up-to-date studies on DNMT1 activity in endometrial healthy and cancer tissues inspired us to commence the study in order to investigate whether there was any relation between DNA methylation and DNMT1 activity as related to the place of collection of the endometrial tissue. In light of the opinions that the process of carcinogenesis in the endometrium preferably begins in utero-tubal junctions, we wished to create a 'map' of DNA methylation and DNMT1 activity within the uterine cavity, which might help to answer the question whether worse endometrium exfoliation in utero-tubal junctions and cervical isthmus is linked to the investigated parameters.

Material and methods

Material

It was a prospective study to investigate the activity pattern of DNMT1 in endometrial cancer. All primary endometrial tumors and normal healthy tissue specimens (n=111) were collected from 37 patients after obtaining an informed consent. Tissue specimens were taken for molecular analyses directly in the operating room, immediately after uterus removal, from three distinct uterine sites: upper to cervical isthmus, uterine cavity, and the utero-tubal junction regions (Figure 1), and stored at -80ºC until DNA extraction. The presence of endometrial adenocarcinoma cells was confirmed in post-operative histological examination at the Department of Pathology, Medical University of Lublin. A total of 84 tissue specimens were taken from 28 patients with endometrial adenocarcinoma – the study (ST) group (ST1; n=39 with histologically confirmed carcinoma cells in samples and ST2; n=45 where no cancerous cells were observed in the samples) and CONT; n=27 samples obtained from 9 patients operated due to symptomatic myomas, where no malignant changes have been described within the endometrium.

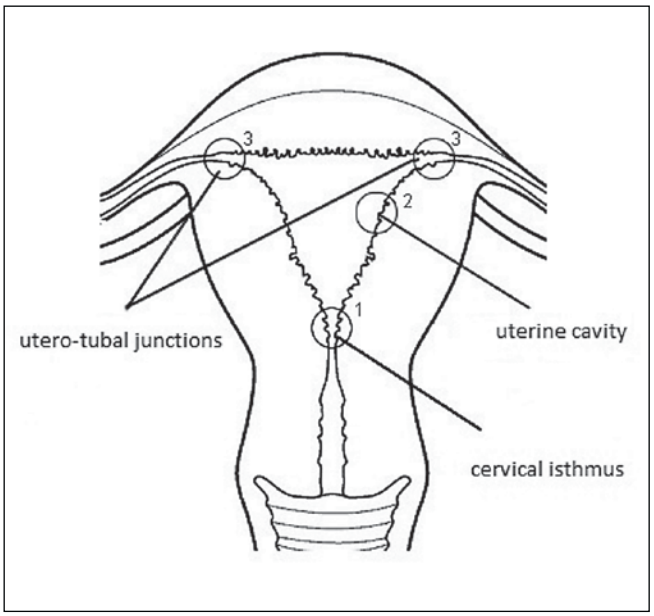

Figure 1. Tissue collection regions.

Methods

DNA has been extracted according to the Chomczynski protocol (Chomczynski, 1987) with the use of commercial TRIzol ® Reagent (Invitrogen) from all of the collected tissue samples. Clearance of DNA samples was checked with the use of biospectrophotometer NanoDrop ND-1000. The samples in which correlation A260/A280 was >1.8 were used for further study. DNA methylation was examined with the use of commercial kits - Methylamp™ Global DNA Methylation Quantification Kit (Epigentek, New York, USA) according to the assay protocol. Separately, proteins have been isolated from all tissue samples using a commercial kit EpiQuik Nuclear Extraction Kit II®. Only samples in which at least 20 µg proteins were extracted were used for further study of DNMT1 activity. Methyltransferase activity was examined with the use of EpiQuik™ DNA Methyltransferase Activity Assay Kit (Epigentek, New York, USA). Statistical analysis was carried out using Statistica StatsSoft, version 9.0. (USA). U-Mann Whitney and W Shapiro-Wilk tests were used. The p-value of <0.05 was considered as statistically significant.

Results

Global DNA methylation in samples in which postoperative histopathology revealed endometrial adenocarcinoma cells was established at 4.51 +/- 1.14%, and was the same as in healthy control tissues (p=0.072). In the endometrial tissue samples from patients with endometrial adenocarcinoma, but in which no cancer cells were found histologically (ST2), DNA methylation levels were approximately the same as in the healthy endometrium (5.15 vs. 5.22%; p=0.065). Significantly lower DNA methylation levels were found in the endometrial cancer samples (ST1) collected from the uterine cavity in comparison to the control group samples obtained from the same area (4.47 vs. 5.72; $p<0.05$) (Figure 2).

Significantly lower DNMT1 activity was observed in the ST1 group as compared to the control group (3.83 OD/h/mg

vs. 7.65 OD/h/mg p=0.0022, respectively), and the ST2 group as compared to the control group (3.24 OD/h/mg vs. 7.65 OD/h/mg p=0.0015, respectively) (Figure 3). Within the ST group, a significantly lower DNMT1 activity was observed in the samples collected from the cervical isthmus in comparison with the CONT group (p=0.019). However, no statistical significance was observed in the endometrial carcinoma samples collected from the tubal junction and the uterine cavity as compared to the healthy samples of the CONT group (p=0.17).

No correlation between DNMT1 enzymatic activity and global DNA methylation was observed in the endometrial adenocarcinoma samples (R=0.077; p=0.64). However, a correlation was observed in the adenocarcinoma samples collected from the tubal junction (R=-0.59; p=0.035).

Discussion

To the best of our knowledge, our study has been one of the first attempts to compare patterns of DNA methylation and enzymatic DNMT1 activity in endometrial adenocarcinoma samples. One can speculate if there are any differences in correlations between DNA methylation pattern and DNMT1 activity in endometrial cancerous and healthy tissue and which mechanisms regulate these differences. The methylation pattern and the methyltransferase activity were examined in three different samples collected from three different locations in the uterine cavity, according to a previously published study, which stated that cancerous cells can excrete hydrogen peroxide which changes oxidation levels in the neighboring healthy tissues or cells [23].

Reports of higher DNMT1 activity levels in healthy endometrial tissue than in endometrial adenocarcinoma observed in our study have never been published. These results can suggest that a study performed with human tissues not always confirms the *in vitro* research. It is well-known that DNMT1 expression is heterogeneous in healthy and cancerous tissues, and no correlation between DNMT1 activity and genome hypomethylation in several human cancers has been reported [24, 25]. Saito et al. [26], confirmed that an increased expression of mRNA DNMT1 can be commenced by chronic inflammation within non-cancerous cells. Endometriosis also can induce increased DNMT1, DNMT3a, DNMT3b expression in relation to normal endometrium [27].

Several publications confirmed increased DNMT1 activity *in vivo* in several human cancers [26, 28, 29, 30, 31]. Ferenczy and Bergeron [32], suggested that the endometrium in contact with cervical isthmus and tubal junction areas, which does not exfoliate completely during the menstrual cycle, is especially prone to cancerous cell transformation. Our results are comparable to the findings of Yajima et al. [33], who reported that endometrial carcinoma appears most often in the 1/3 upper part of the uterus. Other study groups showed that global DNA methylation level can differ, depending on histological tumor advancement and DNA hypomethylation which provokes the oncogenesis process. Epigenetic changes of the genome methylation pattern in type I endometrial cancer are more frequently described in comparison with type II, where DNMT1 activity was decreased [34, 35].

It is important to focus further studies on molecular changes in endometrial cancer to facilitate molecular profiling of each cancer, leading to adequate post-operative treatment.

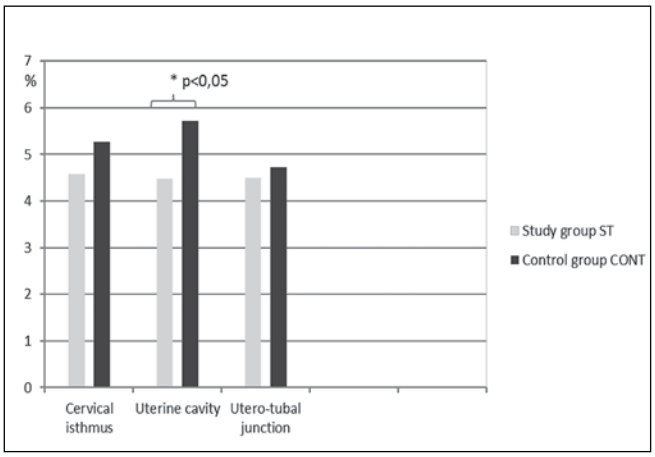

Figure 2. Changes of DNA methylation levels in endometrial carcinoma samples and control sample as related to extraction place from uterine cavity.

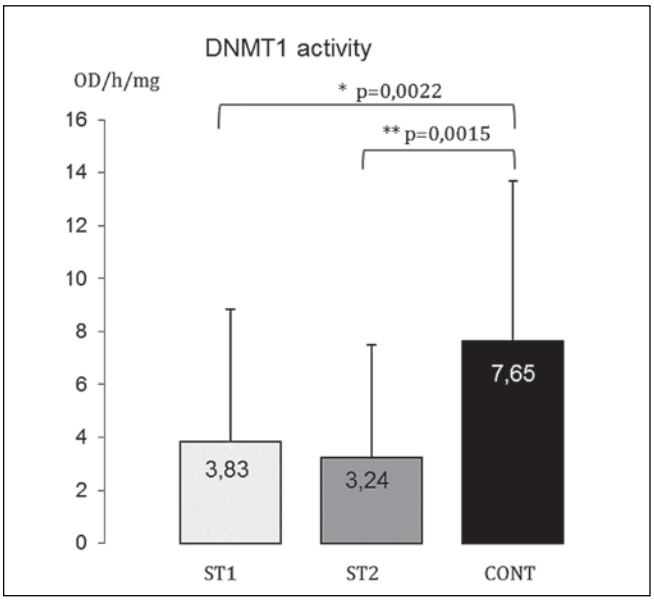

Figure 3. Medium DNMT1 activity in endometrial samples in ST1, ST2 and CONT group.

Conclusions

1. Global DNA methylation remained stable and no significant changes were observed in the endometrial adenocarcinoma samples as compared to healthy endometrial tissues.
2. The enzymatic activity of DNMT1 was significantly lower in endometrial cancer tissue as compared to the healthy endometrium. The lowest activity was observed in adenocarcinoma collected from the cervical isthmus. Research on DNMT1 activity needs further randomized studies with larger sample size to confirm its utility as a potential molecular marker of endometrial adenocarcinoma.

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Oświadczenie autorów:

1. Katarzyna Romanek-Piva - autor koncepcji i założeń pracy, zebranie materiału, realizacja badań laboratoryjnych, przygotowanie manuskryptu i piśmiennictwa – autor zgłaszający i odpowiedzialny za manuskrypt.
2. Krzysztof Gałczyński - zebranie materiału, przygotowanie piśmiennictwa.
3. Aneta Adamiak-Godlewska - współautor tekstu pracy, współautor protokołu, korekta i aktualizacja literatury.
4. Tomasz Rechberger - autor założeń pracy, analizy i interpretacji wyników, przygotowanie, korekta i akceptacja ostatecznego kształtu manuskryptu.
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