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Is global DNA methylation in sporadic uterine adenocarcinomas in women a result of histological and clinical tumor advancement?

Czy globalna metylacja DNA w gruczolakorakach *endometrium* u kobiet jest zależna od histologicznego i klinicznego zaawansowania nowotworu?

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Abstract

Aim: *to find out the relationship, if any, between the extent of the overall genomic DNA methylation, and clinical and pathological features of the sporadic endometrial adenocarcinomas in women.*

Material: *genomic DNA was isolated from 44 primary uterine cancer tissue specimens. There were eight G_1, 24 G_2 and twelve G_3 tumors.*

Methods: *m^5dC level was estimated after enzymatic digestion of DNA into nucleotides, ^{32}P-postlabelling, two-dimensional thin-layer chromatography on cellulose plates and phosphorobioimaging. The overall m^5dC of the uterine cancer DNAs expressed as a ratio: ($pm^5dC/pm^5dC+pdC$) x 100% was compared to results obtained for parallel investigated but published earlier normal human endometrium DNAs.*

Results: *mean total cancer DNA methylation (3.48±0.46%) was significantly higher than that of the normal proliferative endometrium (2.94±0,4%, p=0.003) and lower than that of the secretory endometrium DNAs (3.75±0,47%, p=0.03). Among all endometrial cancer DNAs six were found to be hypomethylated, eight were hypermethylated, whereas the remaining 30 had m^5dC within range of normal endometrium. Total DNA methylation was significantly higher in poorly differentiated (G_3) than in lower grade neoplasms (3.94±0.46 vs. 3.3 ±0.32 %, p=0.025). Lower levels of DNA methylation seemed to be associated with diminished tumor invasiveness.*

Conclusions: *our results suggest that alterations in overall DNA methylation seem to be a result of neoplastic transformation and could therefore be used as a prognostic molecular marker of endometrial cancer.*

Key words: **adenocarcinoma** / **DNA methylation** / / **molecular epidemiology** /

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Streszczenie

Cel pracy: *poszukiwanie zależności między poziomem globalnej metylacji DNA, a niektórymi histologicznymi i klinicznymi cechami gruczolakoraków błony śluzowej macicy u kobiet.*

Materiał i metody: *odsetek 5-metylocytozyny (m^5dC) zbadano przy zastosowaniu metody ^{32}P-postlabeling oraz dwukierunkowej chromatografii cienkowarstwowej (TLC 2-D) z kwantyfikacją radioaktywności nukleozasad przy zastosowaniu czytnika BAS 2000 w próbkach wysokooczyszczonych preparatów genomowego DNA wyizolowanego z 44 guzów (ośmiu w stopniu G_1, 24 G_2 i dwunastu G_3). Wyliczony według wzoru: [(m^5dC/ m^5dC+dC) x 100] poziom zmodyfikowanej zasady korelowano z wiekiem chorych, odróżnicowaniem mikroskopowym guza oraz głębokością naciekania błony mięśniowej ścian macicy przez nowotwór. Poziom globalnej metylacji DNA w nowotworach przeanalizowano ponadto w relacji do uzyskanych równolegle, ale już opublikowanych, wyników badań stopnia metylacji w prawidłowym endometrium.*

Wyniki: *średni poziom globalnej metylacji DNA w gruczolakorakach endometrium (3,48±0,46%) był wyższy niż zanotowano we wzrostowej (p= 0,003) i niższy niż stwierdzono w wydzielniczej błonie śluzowej macicy (p=0,03). Hipometylację stwierdzono w 6, podczas gdy hipermetylacja genomowego DNA wystąpiła w ośmiu nowotworach. Średnia, globalna metylacja guzów w stopniu G_3 była wyższa niż odnotowano w pozostałych (3,94±0,46 vs 3,3±0.32 %, p=0,025). Stwierdzono narastanie poziomu zmodyfikowanego DNA w relacji do stopnia naciekania ścian macicy przez nowotwór.*

Wnioski: *uzyskane wyniki wskazują, że zmiany metylacji cytozyny mogą być raczej skutkiem stopnia transformacji nowotworowej endometrium u kobiet, a nie przyczyną wystąpienia gruczolakoraka.*

Słowa kluczowe: **gruczolakorak** / **metylacja DNA** / / **epidemiologia molekularna** /

Introduction

DNA methylation is an enzymatic, epigenetic modification, which is restricted to cytosine residues at carbon-5 of the pyrimidine ring [1]. Recent evidences implicate the DNA methylation in the regulation of gene expression, in genomic imprinting, in inherited diseases (e.g. fragile-X syndrome), in distinguishing host DNA from foreign DNA, and in oncogenesis [2, 3].

Alterations in DNA methylation are among the most common and earliest events associated with neoplasia [1]. It has been generally accepted that total 5-methyldeoxycytosine content is decreased in most human cancers [4]. Genomic DNA demethylation is involved in increased mutation rate, in increased expression of oncogenes, and finally in tumorigenesis. Counts and Goodman have suggested that only moderate demethylation plays a critical role in carcinogenesis and that excessive hypomethylation could destroy the malignant cells [5]. Gama-Sosa et al. showed that 53% of primary malignancies exhibited lower m^5dC content as compared with normal tissues and benign tumors [6]. They also noted large differences in overall DNA methylation in the same histological type of neoplasm, potentially due to grade of the tumor or contamination of the investigated sample with the normal cells. Bernardino et al. believe that variations of hypomethylation, which is a consistent characteristic of breast cancer, may not correlate with tumor progression [7].

However, Soares et al. found that in such cancer there is a statistically significant correlation between the global DNA methylation and the disease stage, tumor size and histological grade of malignant neoplasms [8].

They also revealed that highest demethylation of DNA was present in G_2 tumors. Shen et al. discovered that total DNA demethylation level is closely correlated with the biological characteristic of liver cancer being more significant in cases with tumor infiltration and metastasis [9].

Moreover, the degree of reduced DNA methylation was related to late histopathological hepatocellular carcinoma (HCC) grade and large tumor size in Lin et al. investigations. These authors also suggest that genome-wide hypomethylation in HCC is the continuing process that persists throughout the lifetime of the tumor cells rather than a historical event occurring at the onset of cancerous growth [10]. Bedford and van Helden did not reveal any change of overall DNA methylation in a case of prostatic cancers spreading by metastasis [11].

Popiela et al. discovered that endometrial cancers in women contain higher grade of total DNA methylation level than those estimated in normal or preneoplastic endometrium [12]. Higher mean DNA methylation level than those revealed in normal tissue was also noted by Kliasheva et al. in cases of human gastric cancers or lung cancer in men [13, 14].

An assessment of the methylation level within neoplastic tissues may provide the basis for future advances in surgical and pharmacologic treatment of human malignant diseases. Staging of cancers on the levels of DNA methylation will provide a convenient way to assess a tumor's biologic aggressiveness and to predict patient outcome [15].

Therefore, the aim of our study was to find out the relationship, if any, between the extent of the overall genomic DNA methylation, and clinical and pathological features of the sporadic endometrial adenocarcinomas in women.

Materials and Methods

Uterine carcinoma samples were obtained from 44 women (age range 34-81), who underwent surgical procedures at the II Department of Gynecology of the Medical University of Lublin, Poland. As a control, normal endometrial samples obtained from 25 regularly cycling, fertile women were used which results concerning total DNA methylation were published earlier [16]. None of the women received any therapy prior to surgery.

Tissues were collected in the operating theater under a dissecting microscope by a punch biopsy of the clearly visible uterine malignancy after dissection of the excised uterus. The tissue samples were immediately frozen in liquid nitrogen and stored at -80ºC until DNA extraction was performed. Each tissue specimen was evaluated at the Department of Pathology. Cancer tissues were classified according to the WHO staging system [17]. The Ethical Committee of the Medical University of Lublin approved the study, and an informed written consent was obtained from each woman. All DNA investigations were accomplished at the Institute de Biologie Moléculaire et Cellulaire, Strasbourg, France.

DNA extraction from tissue samples and estimation of m^5dC level after enzymatic digestion of DNA into nucleotides, ^{32}P-postlabelling, two-dimensional thin layer chromatography on cellulose plates was performed as described earlier [16, 18].
The radioactivity of the labeled spots containing [^{32}P] pm^5dC and [^{32}P] pdC was measured by bio-imaging analyzer (BAS 2000, Fuji). The pm^5dC content was expressed as a ratio: (pm^5dC/ pm^5dC+pdC) x 100%. Each value was calculated from counting of two separate DNA preparations of each individual tissue specimen.

Statistical analysis was performed using the Statistica 8 package. Descriptive statistics were summarized by calculating mean values, standard deviations and ranges. The comparison of m^5dC content between tissues groups was based on a non-parametric Mann-Whitney U test or Wald-Wolfowitz Runs test, when appropriate. A p value less than 0.05 was considered as significant.

Results

We have previously demonstrated that the overall DNA methylation in the normal human endometrium depends on the menstrual cycle phase [16]. DNA methylation was significantly lower in the proliferative than in the secretory endometrium samples. Therefore, we have compared the results of DNA m^5dC content in neoplastic tissues to DNA methylation in normal proliferative and secretory endometrium separately.

The overall content m^5dC in the uterine cancer DNA was 3.48%±0.46 %.

As compared to the normal endometrium, this value was significantly higher than the total m^5dC in the proliferative endometrium (p=0.003), but it was significantly lower than in DNA from the secretory endometrium (p=0.03).

The individual uterine cancer DNAs were considered hypomethylated if the m^5dC values were below the mean for normal proliferative endometrium (2.94%±0.40%), or hypermethylated when the percentage of the m^5dC was above the mean value of the secretory endometrium (3.75%±0.47%).

There were six (14%) hypomethylated and eight (18%) hypermethylated uterine cancer samples. The levels of m^5dC in the remaining 30 (68%) neoplasms were within the range of the normal proliferative and secretory endometrium specimens.
Within neoplasms exhibiting m^5dC level in the range of the normal endometrium samples the most frequent were G_2 tumors (50%), whereas the percentages of G_1 and G_3 tumors were nearly the same (27% vs. 23%, respectively). The percentage of G_2 uterine cancers was not changed in hypomethylated neoplasms, while the percentage of G_1 declined to 17% and the percentage of

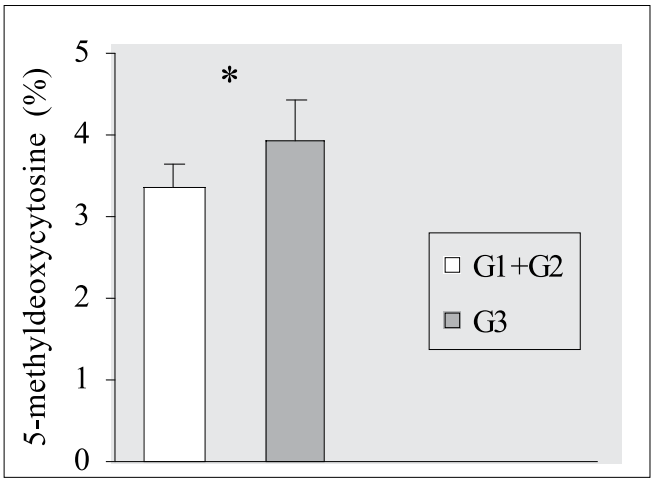

Figure 1. Mean overall m^5dC in less dedifferentiated (G_1 and G_2) and G_3 sporadic adenocarcinomas in women (* - p<0.05).

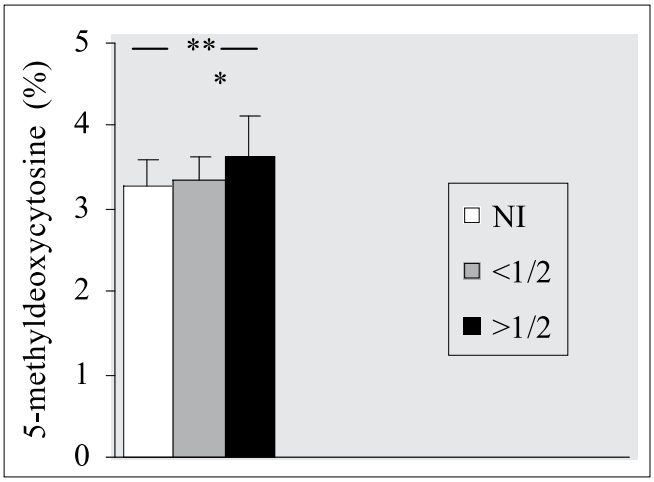

Figure 2. Mean total m^5dC content in endometrial adenocarcinomas according to infiltration of myometrium: NI - cancer confined to endometrial mucosa, <1/2- infiltration less than one half of uterine wall, >1/2- infiltration more than one half of uterine wall thickness.
*- p<0.05; **- p<0.04 (Wald-Wolfowitz Runs test).

G_3 adenocarcinomas increased to 33%. Hypermethylated DNA was present only in G_3 (75%) and G_2 (25%) tumors.

In all uterine cancers investigated the overall DNA methylation of lower histological grade neoplasms (G_1 and G_2) was significantly lower than that of G_3 tumors (3.3±0.3 vs. 3.94±0.46 %, p<0.05). (Figure 1).

Most of the G_1 and G_2 neoplasms (66%) displaying lower values of methylated DNA did not invade or infiltrate the uterine wall more than one half of its thickness. In contrast, the neoplasms with hypermethylated DNA were all associated with deep myometrial invasion, whereas in hypomethylated group it was observed only in 50% cases (3 neoplasms). In cancers confined only to endometrial mucosa (n=4) mean 5-methyldeoxycytosine content was lower than was estimated in tumors invading more

than one half of uterine wall thickness (3.29±0.33 vs. 3.62±0.55%, p<0,05). Higher mean of m^5dC level in such cancers in relation to adenocarcinomas displaying lower myometrial infiltration (3.33±0.29%) was recognized only by applying Wald-Wolfowitz runs test (p<0.04). (Figure 2).

Discussion

Our investigations in a sporadic endometrial carcinomas demonstrated that the mean global DNA methylation was in the range of normal endometrium. Only six uterine cancers exhibited hypomethylation of their DNAs compared to the range of normal endometrium. Additionally, there were no values lower than 2.2%, which was recognized as the lowest value in a DNA sample of normal proliferative endometrium. These results indicate that excessive demethylation of the genome which would be expected to inhibit tumorigenesis, does not take place in human uterine cancer [19]. Alternatively, the data suggest that in endometrial carcinoma hypermethylation of the genome is more common event than demethylation. Moreover, hypermethylation of DNA, which was not observed in the well-differentiated tumors but was mainly attributed to poorly-differentiated neoplasms and some G_2 cancers, may be a molecular marker of high-risk lesions exhibiting the highest rates of solid growth. However, in such tumors we also observed both hypomethylation as well as normal values of DNA m^5dC.

According to presented results, it seems that normal or low DNA methylation status could indicate a low risk endometrial carcinoma, because 75% of the tumors exhibiting normal or decreased values of methylated DNA were G_1 or G_2 neoplasms which are believed as less aggressive tumors [20].

Our results could also clearly indicate that lower total DNA methylation is associated with diminished myometrial invasiveness of the uterine cancer which was recognized as a good prognostic factor of the disease [20].

The extent of DNA methylation in endometrial cancer was significantly higher in G_3 than in G_2 and G_1 neoplasms containing >50%, 6%-50% and <5% of undifferentiated, solid neoplastic cells, respectively [16]. These data support the conclusion that changes in the global level of methylated cytosine are related to the quantity of nonsquamous solid growth. Indeed, about 90% of the G_3 uterine cancers had DNA m^5dC levels higher than the mean content of m^5dC in lower histological grade tumors.

Although local changes in DNA methylation precede malignancy, our results indicate that the global changes appear to be dependent on the histological grade or invasiveness of the endometrial neoplasms [21]. Thus, it appears that increases in global DNA methylation are a result of neoplastic transformation. This conclusion can be supported by fact, that in our investigations the overall DNA methylation in human uterine sporadic cancers was not related to the age of the affected women. This is in contrast to the regional changes of m^5dC content in colorectal cancer tissue, in which aging appears to be a major contributing factor to DNA hypermethylation, but of certain gene loci [22].

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