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8-oxo-7,8-dihydroguanine level – the DNA oxidative stress marker - recognized by fluorescence image analysis in sporadic uterine adenocarcinomas in women

Poziom 8-oksy-7,8-dwuhydroguaniny, markera stresu oksydacyjnego, oceniany metodą fluorescencji w DNA sporadycznych gruczolakoraków błony śluzowej macicy u kobiet

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Abstract

Objectives: *In the case of carcinogenesis in human endometrium no information exists on tissue concentration of 8-oxo-7,8-dihydroguanine, the DNA oxidative stress marker. This was the main reason to undertake the investigation of this DNA modification in human uterine estrogen-dependent tissue cancers.*

Material and Methods: *In order to estimate the level of oxidative damage, 8-oxo-7,8-dihydroguanine was determined directly in cells of tissue microscope slides using OxyDNA Assay Kit, Fluorometric. Cells were investigated under confocal microscope. Images of individual cells were captured by computer-interfaced digital photography and analyzed for fluorescence intensities (continuous inverted 8-bit gray-scale = 0 [black]-255 [white]). Fluorescence scores were calculated for each of 13 normal endometrial samples and 31 uterine adenocarcinoma specimens. Finally, the level of the oxidative stress marker was also analyzed according to histological and clinical features of the neoplasms.*

Results: *The obtained data revealed that: 8-oxo-7,8-dihydroguanine levels were higher in uterine adenocarcinomas than in normal endometrial samples (48,32 vs. 38,64; p<0,001); in contrast to normal endometrium there was no correlation between age and DNA oxidative modification content in uterine cancer; highest mean fluorescence intensity was recognized in G2 endometrial adenocarcinomas; level of 8-oxo-7,8-dihydroguanine does not depend on Body Mass Index (BMI) and cancer uterine wall infiltration or tumor FIGO stage.*

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Conclusions: *Our study indicates that accumulation of the oxidized DNA base may contribute to the development of endometrial neoplasia, however, oxidative DNA damage does not seem to increase with tumor progression.*

Key words: **oxidative stress** / **8-oxoguanine** / **sporadic uterine adenocarcinomas** / / **fluorescence image analysis** /

Streszczenie

Materiał i metody: *Stosując komercyjny zestaw OxyDNA Assay Kit, Fluorometric, przy użyciu mikroskopu konfokalnego oceniono w jednostkach 8-bitowej skali szarości natężenie fluorescencji generowanej przez 8-oksy-7-8 dwuhydroguaninę jąder komórkowych gruczolakoraka błony śluzowej macicy u kobiet.*

Wyniki: *Jądra komórek utkania nowotworowego w relacji do jąder komórek z prawidłowej błony śluzowej macicy zawierały wyższy poziom markera stresu oksydacyjnego. W przeciwieństwie do prawidłowego endometrium, w tkankach nowotworu nie zanotowano korelacji między wiekiem chorej a poziomem 8-oksy-7-8 dwuhydroguaniny, który był najwyższy w nowotworach w stopniu G2. Poziom markera nie był zależny od indeksu BMI, głębokości naciekania ściany macicy przez nowotwór, czy też stopnia FIGO.*

Wnioski: *Przeprowadzone badania wskazują, że stres oksydacyjny DNA może brać udział w nowotworzeniu w endometrium, jakkolwiek jego nasilenie prawdopodobnie nie jest uzależnione od progresji guza.*

Słowa kluczowe: **stres oksydacyjny** / **8-oksyguanina** / **sporadyczne gruczolakoraki błony śluzowej macicy** / **fluorescencyjna analiza obrazu** /

Introduction

Endometrial cancer is the most frequently recognized malignancy of the female genital tract [1]. Most of diagnosed uterine tumors are adenocarcinomas which are believed to be estrogen-dependent [2]. It is widely accepted that in human uterine or mammary hormone-dependent carcinogenesis estrogens act not only as tumor promoters but also as carcinogens [3, 4]. Such a steroid carcinogenic action is strongly connected with metabolism of catechols/*o*-quinones which is a source of overwhelming amounts of reactive oxygen species (ROS) that generate DNA oxidized lesions which, in turn, may lead to gene mutation, and in the next steps, to neoplastic cell transformation [4].

Among more than 30 oxidative DNA adducts that have been characterized, one is 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), and its corresponding base is 8-oxo-guanine which is suspected to be a factor that contributes to cancer by genomic instability increase [5, 6, 7]. It was recognized that 8-oxodG could be a source of GC→TA transversions in the *ras* oncogene and the *p53* tumor suppressor gene in lung and liver cancers [8]. Such mutations were recognized in endometrial adenocarcinomas in 10-30% and 5%, respectively [9, 10].

It has also been postulated recently that 8-oxoG DNA lesion located adjacent to the target cytosine may markedly diminish productive versus nonproductive binding of DNA (cytosine-5-)-methyltransferase 3 alpha to DNA, thus favoring formation of aberrant DNA methylation patterns during carcinogenesis [7].

In estrogen-dependent tissues the rate of oxidative DNA damage may be probably increased by estrogen receptor "Trojan horse" mechanism [11, 12]. This mode of action possibly involves translocation of catechol forms of estrogens and/or *o*-quinones bound to estrogen receptors to steroid sensitive genes where DNA damage occurs resulting in mutations. Indeed, Musarrat et al. [13] revealed that in human breast carcinogenesis estrogen receptor-positive neoplastic tissue contained a significantly higher level of 8-oxodG than in estrogen receptor-negative cancer specimens. Furthermore, in this research 8-oxodG was found to be positively correlated with the amount of estrogen binding sites.

It was also suggested that high levels of oxidative-stress derived DNA lesions preceded oncogenic transformation [3]. Such relationship was revealed by Shimoda's group in humans where liver tissue with chronic hepatitis, which may lead to hepatocellular carcinoma, contained significantly more 8-oxodG than normal livers [14]. A higher amount of 8-oxodG adduct in uterine myomas that may precede uterine sarcomas, in contrast to disease-free surgical margin, was recognized in women by Foksinski et al. [15]. Moreover, they discovered that 8-oxodG levels were proportional to tumor volume.

In addition to this, Ohno et al. revealed that distribution of 8-oxo-7,8-dihydroguanine in the steady-state correlates with the preferred regions for recombination and single polymorphism in the human genome and**,** according to the opinion presented by this group of authors**,** oxidized DNA adduct contributes to the genomic diversity in human beings [16].

In human endometrium carcinogenesis no information exists on tissue concentration of 8-oxo-7,8-dihydroguanine which is recognized as DNA oxidative stress marker. This was the main reason to undertake the investigation of this DNA modification in human uterine estrogen-dependent tissue cancers. Using fluorometric method oxidized DNA lesions were quantified in 13 normal endometrial samples and 31 uterine adenocarcinoma specimens. Finally, the level of 8-oxoGua was also analyzed according to histological and clinical features of the neoplasms. The methods used enabled us to recognize specific patterns of 8-oxo-7,8-dihydroguanine distribution within the investigated cells nuclei, as well.

Materials and methods

The study group consisted of 31 uterine adenocarcinoma patients who underwent total abdominal hysterectomy and bilateral salpingo-oophorectomy at the 2nd Department of Gynecology, Medical University of Lublin, Lublin, Poland. A written informed consent was obtained in each case enrolled in this study. The age range of investigated women was 43-85 years (mean ±SEM 63.6±1.7). All cases were considered sporadic since none of cancer affected women met the Amsterdam criteria [17] for hereditary non-polyposis colon cancer syndrome. Histopathological examination of each collected neoplasm tissue specimen was performed at Pathology Department according to WHO staging system [18].

All specimens were assessed as endometrioid-type endometrial carcinomas. There were 12 well-differentiated (grade 1), 14 moderately-differentiated (grade 2) and 5 poorly-differentiated (grade 3) tumors. Seventeen neoplasms infiltrated uterine wall for less than half of its thickness, whereas 14 cancers displayed deeper myometrial invasion. Nineteen tumors were classified as stage I , nine as stage II and three as stage III according to FIGO surgical staging system [19]. For further analysis uterine cancers categorized into stage II and III were combined as clinically more advanced adenocarcinomas.

The control group consisted of 13 normal endometrial tissue specimens obtained during total abdominal hysterectomy performed due to benign uterine lesions. The mean age of this group was 44.5±2.4 (SEM) (range 32-50).

After uterus removal, the uterine corpus was gently cut from the fundus toward internal os of cervical canal to avoid any contamination of uterine cavity with endocervical cells. Opened uterine cavity was then washed twice with cold (4 ˚C) physiological saline solution to remove blood clots or uterine discharge. The tissue material was then immediately collected into sterile Eppendorf tubes under loupe magnification (5x) using punch biopsy forceps. All tissue samples were quickly frozen and stored in liquid nitrogen.

8-oxo-7,8-dihydroguanine determination

Estimation of 8-oxo-7,8-dihydroguanine was performed using OxyDNA Assay Kit, Fluorometric (Cat. No. 500095) supplied by Calbiochem.

In the first step of analysis tissue specimens were cut at 8 µm thin-sections and subjected to adhesion onto silanized glass slides (DakoCytomation) by baking (16 h, 56 ˚C). Tissue slides rehydrated through serial bath going from absolute to 70% ethanol solution were then placed in 0.2 N HCl and, after washing with distilled water, were further incubated for 30 min in preheated to 80 ˚C Pretreatment Solution supplied to Paraffin Pretreatment Kit (Vysis). After incubation, tissue slides were treated for next 30 min at 37 ˚C with protease solution. Further, tissue sections were fixed in 4% formaldehyde for 10 min at room temperature and after rinsing in Rinsing Buffer (Calbiochem) were dried at 45-50°C for 5 min.

In the next step tissue slides were dehydrated by dipping in cooled (-20°C) solutions containing increasing concentrations (from 70 to 99%) of methanol and after rinsing in 500 µl of Rinsing Solution specimens were subjected to incubation with Blocking Solution. That step eliminates non-specific binding of FITC-Conjugate containing 8-oxo-7,8-dihydroguanine monoclonal antibody conjugated to fluorescein isothiocyanate which was added onto tissue slides in the next step of procedure. After removal of FITC conjugate and rinsing in distilled water (5x) all slides were covered with a glass microscope coverslip.

For each prepared tissue slide, images of 25 randomly chosen nuclei located on slide's diagonal were captured using confocal microscope LSM 5 PASCAL (Zeiss, Germany) equipped with the software version 3.0.

To quantify 8-oxo-7,8-dihydroguanine amount in every investigated nucleus, we cut eleven optical slices, every single separated by 0.8 µm, using 2000x magnification (objective 100x, Immersion-Oil, Apochromat, and digital crop 2x; Figure 1, Figure 2).

All captured images of investigated nuclei were analyzed for fluorescent intensity in an 8-bit grey-scale unit using ImageJ software version 1.33a (Wayne Rasband NIH, USA). The mean value of background fluorescence, i. e. generated by areas located in the vicinity of the investigated nucleus, was subtracted from the total value of each nucleus fluorescent intensity to get a real value of 8-oxo-7,8-dihydroguanine signal.

Descriptive statistics, mean, median, standard error of mean and range values were calculated from the units of fluorescent intensity expressed in an 8-bit grey-scale. An individual level of fluorescence of each tissue slide corresponds to a mean calculated from the total amount of fluorescent intensity of 25 investigated nuclei as described above. The difference between means was calculated using u Mann-Whitney test. Correlation analyses were performed by applying the Spearman's rank correlation test. Statistica 9 software for Windows [StatSoft, Inc. (2009). STATISTICA (data analysis software system), version 9.0. www.statsoft.com.] was used and the level of significance was set at $p<0.05$. Data are expressed as means ±SEM.

Results

Our investigation revealed the presence of 8-oxo-7,8-dihydroguanine in both normal and neoplasmatic endometrial tissue. There were interindividual substantial differences in the amount of oxidized base not only in adenocarcinomas but also in disease-free tissue specimens as indicated by range and median level of fluorescent intensity (Table I).

The highest variations of 8-oxo-7,8-dihydroguanine level were noted in secretory endometrium in which the maximum value of fluorescence was 10 fold higher than the lowest one (7.98 vs. 79.19). In contrast, in adenocarcinomas group that value was amounted to about 4 (31.92 vs. 126.9) and was the highest in G2 tumors. However, the lowest value of fluorescent intensity in cancers was about 3 fold higher than it was revealed in noncancerous endometrial tissue (7.98 vs. 23.93).

Table I. Range and median level in units of [8-bit] grey-scale of 8-oxo-7,8-dihydroguanine in human endometrium.

Endometrial histology	range	median
Normal endometrium n=13	7.98 – 79.19	32.9
Adenocarcinoma n=31	23.93 – 126.9	39.05

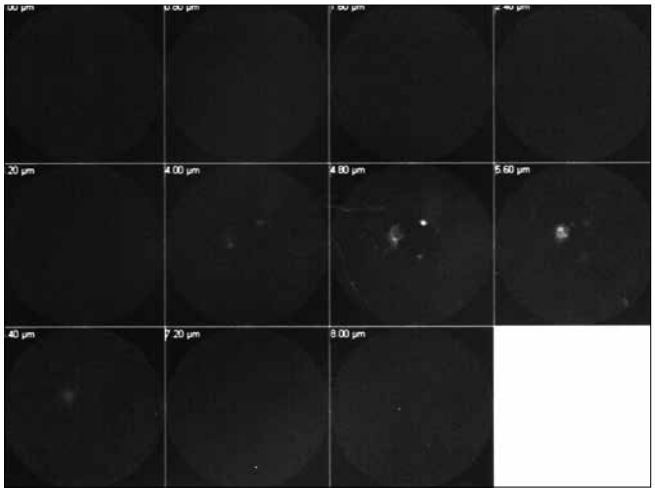

Figure 1. Digital photography of 11 optical slices of human normal endometrium nucleus (2000x magnification). White areas reflect 8-oxo-7,8-dihydroguanine DNA lesion distribution.

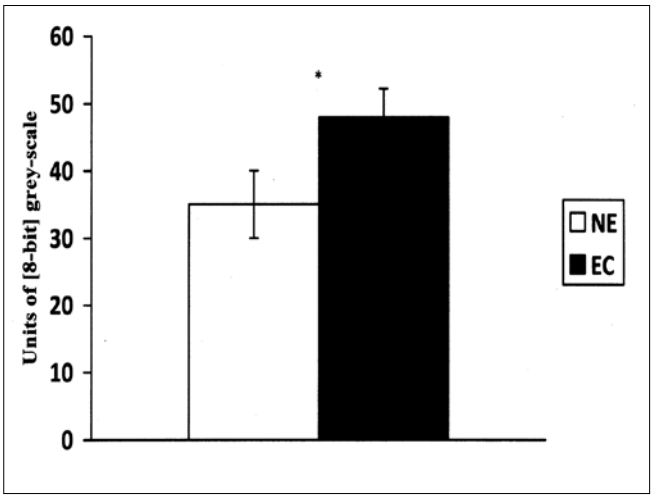

Figure 3. Mean amount (±SEM) of 8-oxo-7,8-dihydroguanine in normal endometrium (NE) and uterine adenocarcinomas (EC); * p<0,001.

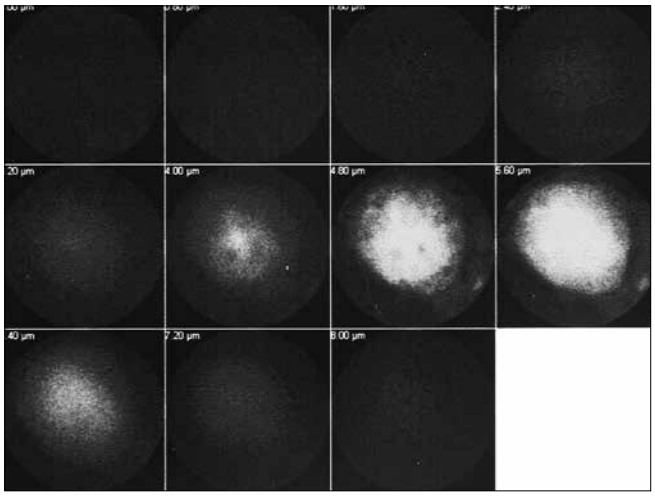

Figure 2. Digital photography of 11 optical slices of human uterine adenocarcinoma tissue nucleus (2000x magnification). White areas reflect 8-oxo-7,8-dihydroguanine DNA damage distribution.

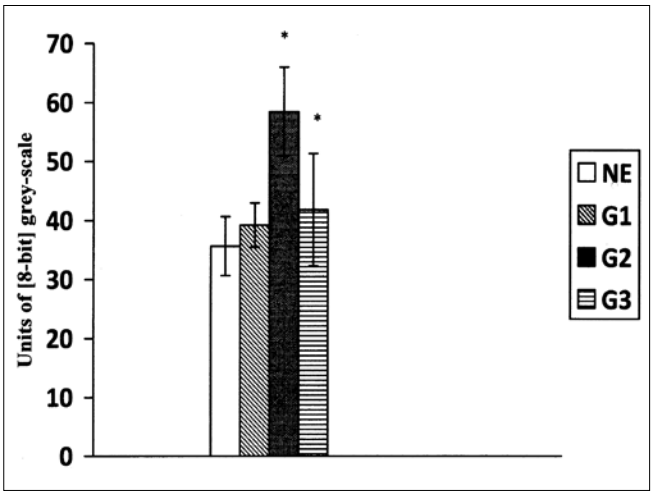

Figure 4. Mean (±SEM) 8-oxo-7,8-dihydroguanine level in uterine adenocarcinomas according to WHO grading system in comparison to normal endometrium (NE); * p<0,001.

There were no differences between mean oxidative DNA adduct level in proliferative and secretory endometrium (37.99±5.16 vs. 37.45±10.1). In normal endometrium there was a significant, positive correlation between DNA 8-oxo-7,8-dihydroguanine level and patient's age along with no relation to BMI (R=0.47, p=0.028; R= -0.04, p=0.85, respectively).

The mean 8-oxoGua amount in uterine adenocarcinomas was significantly higher than it was recognized in normal endometrium (48.32±4.23 vs. 35.66±5.01; p<0.001), (Figure 3). There were no correlations between 8-oxoGua content and age or BMI of cancer affected women.

A mean oxidized DNA adduct level in cancers divided according to WHO classification was higher than in noncancerous endometrium in every tumor grade group, however in G1 adenocarcinomas it was not statistically significant (Figure 4).

The highest mean 8-oxo-7,8-dihydroguanine level was recognized in G2 neoplasms (58.41±7.56). That was significantly higher than it was revealed in G1 (39.24±3.71) and G3 (41.81±9.5) tumors (p<0.0001; p<0.000002, respectively). A mean 8-oxoGua amount in G3 neoplasms was higher than it was recognized in G1 endometrial cancers (p<0.0001). There were no differences in 8-oxo-7,8-dihydroguanine amount between tumors confined to endometrial mucosa or invading myometrium for less than half of its thickness and neoplasms displaying deeper uterine wall invasion (50.78±6.05 vs. 45.32±5.97; p=0.32). In uterine adenocarcinomas classified according to FIGO as stage I tumors a mean level of oxidized guanine was comparable to the one recognized in more clinically advanced neoplasms (46.73±5.62 vs. 50.82±6.58; p=0.72).

As far as 8-oxo-7,8-dihydroguanine distribution within cell nucleus is concerned type A image was noted only in normal endometrium and to a lesser degree in tumor tissue, whereas type B, C and D were merely present in adenocarcinomas (Figure 5).

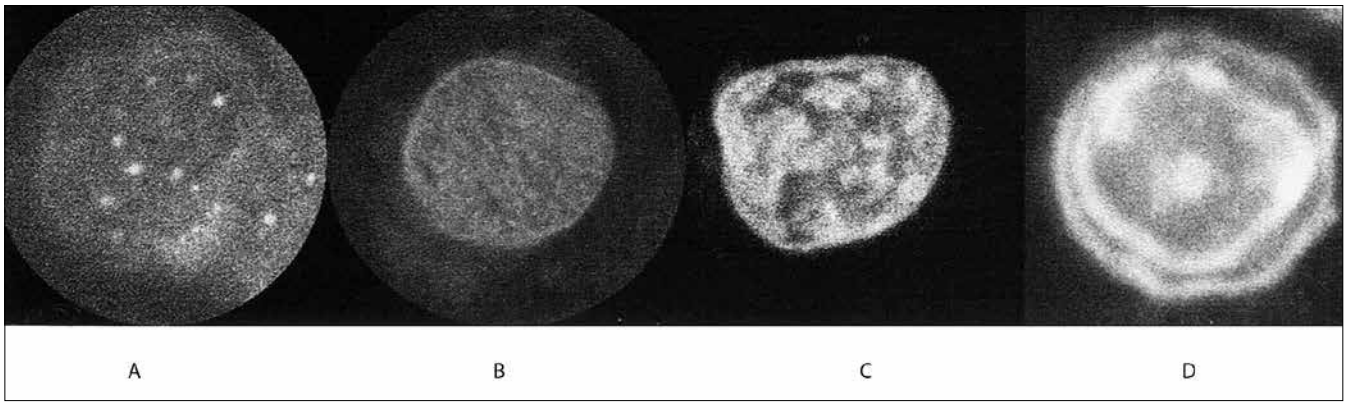

Figure 5. 8-oxo-7,8-dihydroguanine distribution within cell nucleus in normal endometrium (A) and adenocarcinomas tumor tissue (B, C, D). Digital image taken under confocal microscope (2000x magnification).

Discussion

This study shows for the first time the levels of 8-oxo-7,8-dihydroguanine in normal endometrium and sporadic uterine adenocarcinomas which are widely known as estrogen-dependent tissues [20]. In the presented study the results of tumors' fluorescence intensity generated by 8-oxo-7,8-dihydroguanine were compared to those obtained in endometrium collected from cancer-free women instead of measurements from margin tissues surrounding the neoplasms. This strategy was based on the opinion that human neoplastic cell lines, including ovarian carcinoma that is suspected to be estrogen-dependent, can release in culture large amounts of hydrogen peroxide that can alter an oxidative level of neighboring disease-free tissues [21, 22].

Therefore, we believe that, as revealed by us, the endometrial concentrations of oxidized bases truly represent the background level of oxidative stress, reflecting also the equilibrium between formation and repair of damaged DNA including 8-oxoGua. According to Serviddio et al. the steady-state level of oxidative stress in human endometrium is also a result of an antioxidant defense system action which involves the glutathione peroxidase (GSH-Px)-related enzymatic reduction of hydrogen peroxide and lipid hydroperoxides by transformation of glutathione (GSH) to its oxidized form (GSSG) [23].

Besides, that group of authors revealed that in normal endometrium of menstruating women GSH-Px activity and GSSG as a percentage of GSH were positively correlated with plasma estradiol or LH concentration. They also discovered that estradiol concentration negatively correlates with GSH. On the other hand, Serviddio's group found no significant relationship between plasma progesterone or FSH concentration and oxidative balance, although the earlier data of Ohwada et al. [24] revealed that GSH-Px endometrial activity in spayed rats treated with progesterone was lower than in untreated controls. Because we did not perform hormonal estimations in our group of women we can only suspect that widely known changes of blood estradiol and LH concentrations along with related to them GSH-Px activity and glutatione concentration could be the reason of observed by us considerable interindividual differences in the amount of 8-oxo-7,8-dihydroguanine i. e. oxidative stress in noncancerous endometrial tissues.

Based on data published by Musarrat et al. [13] that oxidative DNA damage correlates with estrogen receptors (ERs) content we expected a higher amount of 8-oxo-7,8-dihydroguanine in proliferative than in secretory endometrium. That tissue, even in premenopausal women, contains a higher ERs concentration and being in this menstrual cycle phase under high estrogen influence should theoretically display higher oxidative DNA changes [25].

However**,** in our investigations 8-oxo-7,8-dihydroguanine level in proliferative endometrium was similar to the one revealed in secretory tissue, although half of women in this group were premenopausal what, according to Gürdöl et al. [26], should implicate lower GSH-Px activity, thus impaired antioxidant defenses. In normal endometrium we were able to recognize a positive correlation between oxidized DNA adducts level and age which is a major risk factor for endometrial cancer. It was in contrast to Matsui et al. [27] who did not find any relationship between the level of 8-OHdG and age in noncancerous breast tissue which, as endometrium, is also believed to be a hormone-dependent tissue.

Our investigations indicate that uterine adenocarcinomas contained a significantly higher level of 8-oxo-7,8-dihydroguanine than cancer-free normal endometrium. Such a relationship between oxidized G content in cancer and cancer-free tissue was also revealed by Malins et al. [28] and Musarrat et al. [13] but in other estrogen-dependent organ like breast. An increase of 8-OHdG in DNA of neoplastic tissue in comparison to neighboring normal tissue was noted by Okamoto et al. [29] in renal-cell carcinoma and Oliva et al. [30] in sporadic colon cancers. However, Kirkali et al. [31] has no found such relationship in human colorectal cancer lately.

Well-differentiated endometrial adenocarcinomas (G1) contained the level of 8-oxoG only slightly higher than control endometrium. Most of these neoplasms (7 out 12) contained concurrent atypical adenomatous hyperplasia and they were confined to endometrial mucosa or invaded myometrium for less than half of its thickness. According to Ohwada et al. [24] such tumors always displayed high levels of GSH-Px activity, thus we believe that in our investigation antioxidant defense system could probably prevent higher oxidized base formation in that series.

The level of 8-oxo-7,8-dihydroguanine also depends on its removal from DNA by DNA repair enzymes and the removal of 8-oxodGMP from the cellular nucleotide pool what prevents its incorporation into DNA. Such mechanism was elegantly described by Olinski's group in non-small-cell lung cancer [32]. However, due to the lack of available data concerning uterine carcinogenesis such a contribution to the maintenance of 8-oxoG levels in endometrial cancer DNA seems only possible.

Moderately-differentiated uterine adenocarcinomas displayed the highest 8-oxoGua that decreased in poorly-differentiated ones. We suspect that such a pattern of oxidized base amount could be due to several reasons including impairment of antioxidant defense system provoked by lowering of estrogen-binding sites which reach their minimum concentration in most histologically dedifferentiated endometrial adenocarcinomas [24, 33]. A decrease of 8-oxoG in G3 as compared to G2 endometrial cancers may also depend on dilution of DNA oxidized adducts by DNA replication during rapid cell turnover in most advanced cancers or an increase of removal of 8-oxodGMP from the cellular nucleotide pool by hMTH1 action. This hydrolase gene expression was increased in human renal-cell carcinoma, and as it was also reported, more advanced-stage tumors showed a higher level of hMTH1 expression [34].

We cannot exclude that 8-oxo-7,8-dihydroguanine level could also be related to contamination of cancer tissue with noncancerous stromal or inflammatory metaplastic cells or lymphocytes which all of them display different oxidative potential [35]. Indeed, Zbar et al. [36] revealed that about 60% of clarocellular type of kidney cancer tissue is composed of lymphocytes, whereas Yamamoto et al. [37] discovered that the quantity of lymphocytes infiltrating uterine neoplastic tumors known as TIL – Tumor Infiltrating Lymphocytes increased proportionally to cancer invasiveness. Nowsheen et al. [38] believed that more histologically homogenous tumor biopsies of liver, kidney or ovarian neoplasms gave even more results of the quantity of oxidatively induced clustered DNA lesions than colon and breast tumor specimens in which different levels of the lesions probably depended on the ratio of cancerous epithelium and normal fibroblasts.

Neither depth of myometrial invasion nor clinical advancement of endometrial adenocarcinomas or BMI and age of the cancer affected women had any effect on 8-oxoG amount in tumor tissue. Sparse data on influence of some histo-clinical features of uterine neoplasms and their risk factors on the oxidized DNA adduct content is a limitation that prevents a final conclusion. Only Matsui et al. [27] reported that in breast cancers other estrogen dependent kind of neoplasms, the 8-oxodG level, was not dependent on lymph node metastasis. However, they were able to recognize negative correlations of this DNA modification content and clinical stage of disease and lymph node status.

We believe that our results that show different distribution of 8-oxoG in nuclei can represent not only the quantitative differences of oxidized adduct but they could probably also reflect the heterogeneity of the investigated tissue specimens. Such possibilities are now under intensive investigation in our laboratories and we will be able to publish our results soon.

Conclusion

Our study indicates that in endometrial adenocarcinomas the level of 8-oxo-7,8-dihydroguanine cannot serve as a marker of the involvement of the disease. It also suggests that accumulation of the oxidized DNA base may contribute to the development of endometrial neoplasia, however, oxidative DNA damage does not seem to increase with tumor progression.

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