VILNIUS UNIVERSITY

Lina Daukantienė

INFLUENCE OF MOLECULAR AND GENETIC FACTORS OF THE ORGANISM´S ANTIOXIDATIVE SYSTEM ON CERVICAL CARCINOGENESIS AND THE ROLE OF THESE FACTORS IN TREATMENT OF LOCALLY ADVANCED CERVICAL CANCER

Summary of Doctoral Dissertation

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VILNIAUS UNIVERSITETAS

Lina Daukantienė

ORGANIZMO ANTIOKSIDACINĖS SISTEMOS MOLEKULINIŲ IR GENETINIŲ VEIKSNIŲ ĮTAKA GIMDOS KAKLELIO KANCEROGENEZEI BEI ŠIŲ VEIKSNIŲ REIKŠMĖ GYDANT VIETIŠKAI IŠPLITUSĮ GIMDOS KAKLELIO VĖŽĮ

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ABBREVIATIONS

AD – average dose

AF – alkaline phosphatase

ALT – alanine transaminase

AST – asparagine transaminase

ChRT – chemoradiotherapy

CI – confidence interval

CIN – cervical intraepithelial neoplasia

CIS – carcinoma *in situ*

CT – computed tomography

CTCAE – Common Terminology Criteria for Adverse Events

DNA – deoxyribonucleic acid

EBRT – external beam radiotherapy

ELISA – enzyme-linked immunosorbent assay

ECOG – Eastern Cooperative Oncology Group

EORTC – European Organization for Research and Treatment of Cancer

FIGO – International Federation of Gynaecology and Obstetrics

FR – free radicals

GSH – reduced glutathione

GST – glutathione- S-transferase

Gy – grey (unit of measurement of ionizing radiation dose)

HP – human papillomavirus

HSIL – high-grade squamous intraepithelial lesions

LSIL – low-grade squamous intraepithelial lesions

NChT – neoadjuvant chemotherapy

p – level of significance

RTOG - Radiation Therapy Oncology Group

SD – standard deviation

SFD – single focal dose

TD – total dose

WHO – World Health Organization

1. INTRODUCTION

1.1. Relevance of the study

Cervical cancer is the fourth most common cancer among women worldwide and the fourth leading cause of death in female population; it is the second most common cancer in young women, also. Annually, more than a half of million of new cases of cervical cancer is being diagnosed in the world, and more than 85% of the cases occur in the less economically developed countries [http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx.]. According to the data of National Cancer Institute, cervical cancer in Lithuania was both the fourth most common cancer and cause of death in female population, in 2011, and it took the second place regarding morbidity and cancer mortality among women aged 30 – 54 years. [http://www.nvi.lt].

A lot of factors, including viral, genetic, ethnic, environmental ones, nutrition habits have an impact on development of cervical cancer [Wang SS, 2009].

The changes in oxidative-reductive system are important for formation of cancer of various localizations, including cervical cancer, as well; these changes are reflected by intensity of lipid peroxidation and activity of antioxidative enzymes. The increase of free radicals (FR) and decrease of ability of antioxidative system to neutralize these radicals result in development of oxidative stress. The excess of FR causes impairment of nuclear acids, initiates lipid peroxidation chain reactions, disturbs intracellular signalling pathways and in this way influences carcinogenesis during the stages of initiation and promotion [Reuter S. et al., 2010].

Oxidative stress in cancer patients can be stimulated and deteriorated not only by cancer itself, but also by radiotherapy and chemotherapy that directly and indirectly increase intracellular level of FR. [Noqueira V. et al., 2013]. On the contrary, the factors reflecting condition of antioxidative system might be significant in predicting the course of the disease and response to the treatment [Huang G. et al., 2007].

Reduced glutathione (GSH) is one of the most important antioxidant and detoxifying agent. GSH regulates action of other antioxidants, detoxifies harmful compounds, modulates activity of immune system and development of inflammatory processes, takes part in transportation of amine acids through cellular membrane, increases solubility of toxic materials or medicines in water by forming conjugates; GSH

is a cofactor of some enzyme reactions, also [Traverso N. et al., 2013]. GSH is used as a substrate by the enzymes, including glutathione S-transferase (GST).

Primary, as well as secondary antioxidative enzymes, including GST taking part in conjugation of toxic compounds to glutathione, are important for the carcinogenesis. On the one hand, with the decrease of GST enzyme activity, the ability of the organism to metabolize carcinogens decreases, also, resulting in increase of the amount of cytogenetic injuries and cancer development risk. On the other hand, the high activity of GST enzymes causes more rapid metabolism of the medicines, resulting in suppression of efficacy of cytotoxic therapy; this effect may be related with poorer response to the treatment and shorter survival duration of the patients [Peklak-Scott C. et al., 2005].

Several studies to evaluate the level of GSH and GST in cervical cancer patients' blood and tumour tissue were performed [Beevi SS. et al., 2007; Martens JE. et al., 1997]. The data of literature reviewed showed that significant differences between subjects suffering from cancer and healthy ones were present. The relationship between differences of GSH and GST blood levels and various clinical characteristics, e.g. the stage of the disease, changes of the GSH and GST levels during the treatment (chemotherapy, radiotherapy) was also stated; this relationship may be important both from the point of view of prediction and prognosis [Sharma A. et al., 2010].

The relationship between GST genes polymorphism and the risk of development of breast, lung, urinary bladder and prostate cancer was ascertained [Di Pietro G. et al., 2010]. The complete loss of GSTM1 and GSTT1 enzyme activity related to homozygous gene deletion was found out in 50% and 25% of Caucasian subjects, respectively [Piacentini S. et al., 2011]. The data regarding the relationship between GST genes polymorphism and the risk of cervical cancer are limited.

So, taking into account the importance of GST enzyme activity and GST genes polymorphism, as well as the GST enzyme substrate GSH, in carcinogenesis and potential prognostic and predictive value of these factors, further studies are reasonable.

Approximately in 50% of the cases locally advanced cancer stage IIB-IVA is diagnosed, despite screening programme and prevention measures performed [http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx., http://www.nvi.lt.]. The standard of treatment of locally advanced cervical cancer is cisplatin-based chemoradiotherapy; however, the results of the treatment are unsatisfactory, as local and distatant relapses are

frequent. Therefore, attempts to find out more effective ways of treatment are taking place, including introduction of new cytostatics and target therapy into clinical practice; clinical studies evaluating combination of radiation therapy and new cytostatics without cisplatin or new cisplatin combinations used as neoadjuvant or adjuvant therapy are being performed; new methods of application of cytostatics, regimens and doses are being tested. The research of new prognostic and predictive markers enabling one to stratify patients in accordance with the aggressiveness of the disease and enabling to choose the optimal way of therapy is going on, in order to individualize the treatment. It is known that the following clinical and pathologic factors have undoubted prognostic value: disease stage, tumour size, metastases of regional lymph nodes, histology type, tumour differentiation grade, lymphatic or vascular invasion, hypoxia, blood haemoglobin level. However, even in event of identical data, the patients' response to the treatment and survival duration differs. That is why further scientific research based on molecular biology and genetics are encouraged.

1.2. Aim of the study

To assess the importance of molecular (reduced glutathione (GSH) and glutathione S-transferase (GST)) and genetic (glutathione S-transferase (GST) genes polymorphism) factors determining the activity of antioxidative system for development of cervical cancer and evaluate the role of these factors during the treatment of locally advanced cervical cancer by means of neoadjuvant chemotherapy and chemoradiotherapy.

1.3. Tasks of the study

1. To estimate the blood serum levels of reduced glutathione (GSH) and glutathione S-transferase (GST) in patients with invasive locally advanced cervical cancer, women with cervical intraepithelial lesions (CIN3/CIS) and healthy women.
2. To assess the distribution rate of glutathione S-transferase genes (*GSTM1* and *GSTT1*) polymorphism in patients with invasive locally advanced cervical cancer, women with cervical intraepithelial lesions (CIN3/CIS) and healthy women.
3. To evaluate the efficacy and safety of the intense neoadjuvant chemotherapy with combination of cisplatin and gemcitabine followed by chemoradiotherapy (using the same combination of cytostatics) for the patients with cervical cancer of IIB – IIIB stages.
4. To assess the changes of reduced glutathione (GSH) and glutathione S-transferase (GST) levels during the treatment and establish the relationship with clinical and pathological characteristics of the cervical cancer, response to the treatment and disease progression.

1.4. Statements to be defended

1 The blood serum levels of reduced glutathione (GSH) and glutathione S-transferase (GST) are different in patients with invasive locally advanced cervical cancer, women with cervical intraepithelial lesions and healthy women. The probability of cervical cancer development is increased in presence of glutathione S-transferase (GSH) genes (*GSTM1* and *GSTT1*) polymorphism.
2. The intense weekly regimen of neoadjuvant chemotherapy with cisplatin and gemcitabine followed by chemoradiotherapy (when the same combination of cytostatics is used) is effective and safe for patients with locally advanced cervical cancer of IIB-IIIB stages.
3. The dynamics of levels of reduced glutathione (GSH) and glutathione S-transferase (GST) during the treatment of locally advanced cervical cancer is significant for predicting the treatment effectiveness.

1.5. Scientific novelty of the study

1. Scientific and clinical studies of oxidative stress in cases of precancerous cervical diseases and cervical cancer are not abundant; the data is not yet systematized. The available studies about the relationship between glutathione S-transferase (GST) genes polymorphism and risk of cervical cancer are controversial; limited data is available on the *GSTM1*, *GSTT1* genes polymorphism influence on the risk of cervical cancer in European populations. In our study statistically significant differences in blood serum levels of reduced glutathione (GSH) and glutathione S-transferase (GST) in patients with locally advanced cervical cancer, women with cervical intraepithelial lesions (CIN3/CIS) and healthy women demonstrated the importance of antioxidative system´s components in cervical carcinogenesis. Taking into account statistically significant incidence of deletions in *GSTM1* and *GSTT1* genes among these groups, it can be stated, that in event of polymorphism of genes mentioned above, the probability to develop cervical cancer is higher.
2. Locally advanced cervical cancer was treated with the new intense neoadjuvant chemotherapy using combination of cisplatin and gemcitabine followed by chemoradiotherapy (when the same combination of cytostatics was used). Little is known about changes of parameters of oxidative stress during the cervical cancer treatment process. Based on the data from our study it might be concluded that changes in GSH levels in the process of the treatment of locally advanced cervical cancer can be significant for predicting of the treatment effectiveness.

2. MATERIAL AND METHODS

The protocol of prospective clinical trial was initiated and developed at the National Cancer Institute (previous Institute of Oncology, Vilnius University) (Protocol No. A7-14). Approval of the study was obtained from Lithuanian Bioethics Committee (2009-10-08 No.P-09-61) and State Medicines Control Agency (2009-11-19 No.12KL-219). The participants had read, understood and signed the Informed Consent Form, prior inclusion into the study.

2.1. Characteristics of the study population

The study included 94 women, who were distributed into 3 groups:

The 1st group included 36 patients, for whom locally advanced International Federation of Gynaecology and Obstetrics (FIGO) stage IIB-IIIB cervical cancer was diagnosed and confirmed histologically; these patients had no history of treatment for the cervical cancer.

The 2nd group included 27 women, for whom cytology test of the cervix revealed high grade squamous intraepithelial lesions (HSIL). All these women underwent conisation of the cervix; cervical intraepithelial neoplasia grade 3/carcinoma *in situ* (CIN3/CIS) was confirmed for all these women histologically.

The 3rd group consisted of 31 healthy volunteer women.

The age and clinical-pathology characteristics of the study subjects are presented in Table 2.1.1. All the subjects maintained that they had no history of smoking, chronic diseases (i.e. diabetes mellitus, rheumatoid arthritis, uncontrolled arterial hypertension) and used no vitamins or other antioxidative preparations.

Table 2.1.1. Distribution of the study subjects according to the age and clinical-pathology characteristics

Characteristics	**Patients with cervical cancer, n = 36, n (%)**	**Patients with cervical intraepithelial lesions, n=27**	**Healthy women, n = 31**
Mean age ± SD, yrs.	47.7 ± 8.08	41.5 ± 9.96	41.1 ± 9.27
FIGO stage: II B III A III B	 17 (47) 1 (3) 18 (50)		
Histological type of tumour: squamous cell carcinoma adenocarcinoma	 33 (92) 3 (8)	 CIN3/ CIS	
Tumour differentiation grade: G1 G2 G3	 4 (11) 6 (17) 26 (72)		
ECOG performance status: ECOG = 0 ECOG = 1	 32 (89) 4 (11)		

n – number of cases, FIGO – International Federation of Gynaecology and Obstetrics, G1 – low grade (well-differentiated tumour), G2 – intermediate grade (moderately differentiated tumour), G3 – high grade (poorly differentiated tumour), ECOG – Eastern Cooperative Oncology Group, CIN/CIS – cervical intraepithelial neoplasia grade 3/ carcinoma *in situ.*

The Protocol of the study included 2 sections:

- Clinical: to perform the new intense weekly neoadjuvant chemotherapy followed by chemoradiotherapy for patients with locally advanced cervical cancer.
- Laboratory: to assess the changes of molecular and genetic factors important for function of antioxidative system, including GST enzyme, polymorphism of its genes and GST substrate GSH in blood serum of patients with locally advanced cervical cancer, women with cervical intraepithelial lesions and healthy women.

2.2. Clinical section of the study

A total of 36 patients were enrolled in the prospective study between 2010 and 2012.

2.2.1. Inclusion criteria of the patients

The study included patients who were 18 – 70 years old, for whom locally advanced stage IIB-IIIB cervical cancer was diagnosed and who had no history of treatment for cervical cancer; the performance status of the patients, according to Eastern Cooperative Oncology Group (ECOG), was ≤ 1; all the participants had signed the Informed Consent Form.

Criteria of non-inclusion or withdrawal from participation in the study included: unstable angina pectoris, myocardial infarction ≤ 6 month prior screening visit, uncompleted supportive treatment for life-threatening cardiac arrhythmia, pulmonary hypertension; laboratory findings: absolute neutrophil count < 1.5 x 10^9/l, platelet count ≤ 100 x·10^9/l, haemoglobin ≤ 90 g/l, blood serum creatinine level ≥ 1.5-fold higher than upper limit of normal, blood serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels ≥ 3-fold higher than upper limit of normal, pregnancy, breastfeeding and serious mental illness.

The period of screening consisted of collection of comprehensive history of life and illness, evaluation of tumour biopsy findings (tumour morphology, tumour differentiation grade), objective evaluation of patient's condition; laboratory tests were also performed during the screening, as well as diagnostic procedures, in order to evaluate tumour stage, including vaginal, rectal and vaginal examinations, radiological (radiography, ultrasound examination, computed tomography (CT)) examination of the

chest, abdomen and pelvis, standard and perfusion CT of the cervix; when invasion into urinary bladder and rectum was suspected, the patients underwent cystoscopy and rectoscopy.

2.2.2. Patient characteristics

The mean age of the patients was 47.7±8.08 years (ranging from 29 to 67 yrs.). Clinical FIGO stage: IIB – 47% (n=17), IIIA – 3% (n=1), IIIB – 50% (n=18) of the patients. CT revealed metastases in regional lymph nodes (the size of lymph node > 10 mm) of the pelvis in 58% (n=21) of the patients and in 42% (n=15) CT showed no metastases in regional pelvic lymph nodes. The following results of tumour histopathology examination were obtained: squamous cell carcinoma was present in 92% (n=33) of the patients and 8% (n=3) of them was suffering from adenocarcinoma. It was found that 11% (n=4) of the patients had tumour of G1 tumour differentiation grade, 17% (n=6) had G2 tumour and in 72% (n=26) G3 tumours were detected. After evaluation of performance status, it was assessed that 89% (n=32) and 11% (n=4) of the patients had ECOG score 0 and 1, respectively.

2.2.3. Methods of treatment and investigations

In accordance with the Protocol of the study, short-term intense (weekly) course of neoadjuvant chemotherapy using combination of cisplatin and gemcitabine was administered for patients with locally advanced cervical cancer. Subsequently, chemoradiotherapy was applied. The chemoradiotherapy treatment included chemotherapy using the combination of cisplatin and gemcitabine, administrated weekly during the period of external beam radiotherapy (EBRT). Intracavitary brachytherapy was given following the completion of EBRT. The scheme of the treatment applied is presented in Fig. 2.2.3.1.

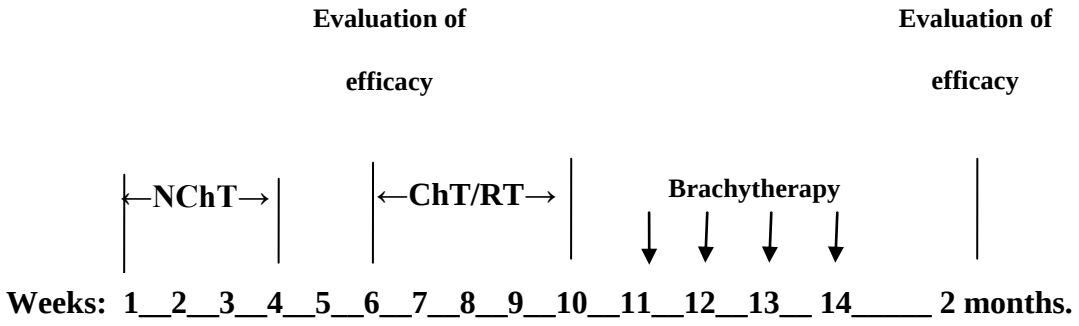


Figure 2.2.3.1. Scheme of the treatment

NChT – neoadjuvant chemotherapy; ChT/RT – chemoradiotherapy.

Patient's condition was evaluated and general haematology test performed every time before chemotherapy; the biochemistry tests were performed every 4 weeks. In event of side effects, standard treatment of these effects was performed.

Neoadjuvant chemotherapy. Neoadjuvant chemotherapy consisted of combination of intravenous cisplatin (30 mg/m²) and gemcitabine (125 mg/m²) once a week for four consecutive weeks. Hydration, infusion duration and antiemetic prescription were administered according to the standard guidelines.

A detailed physical examination, including a pelvic examination, standard and perfusion CT scan were performed 14 days after completion of the neoadjuvant chemotherapy, in order to evaluate the efficacy of the treatment. Images taken from CT with immobilization tools were used to plan EBRT and brachytherapy. A three-dimensional conformal EBRT dosimetry plan (3D) was arranged (Fig. 2.2.3.2).

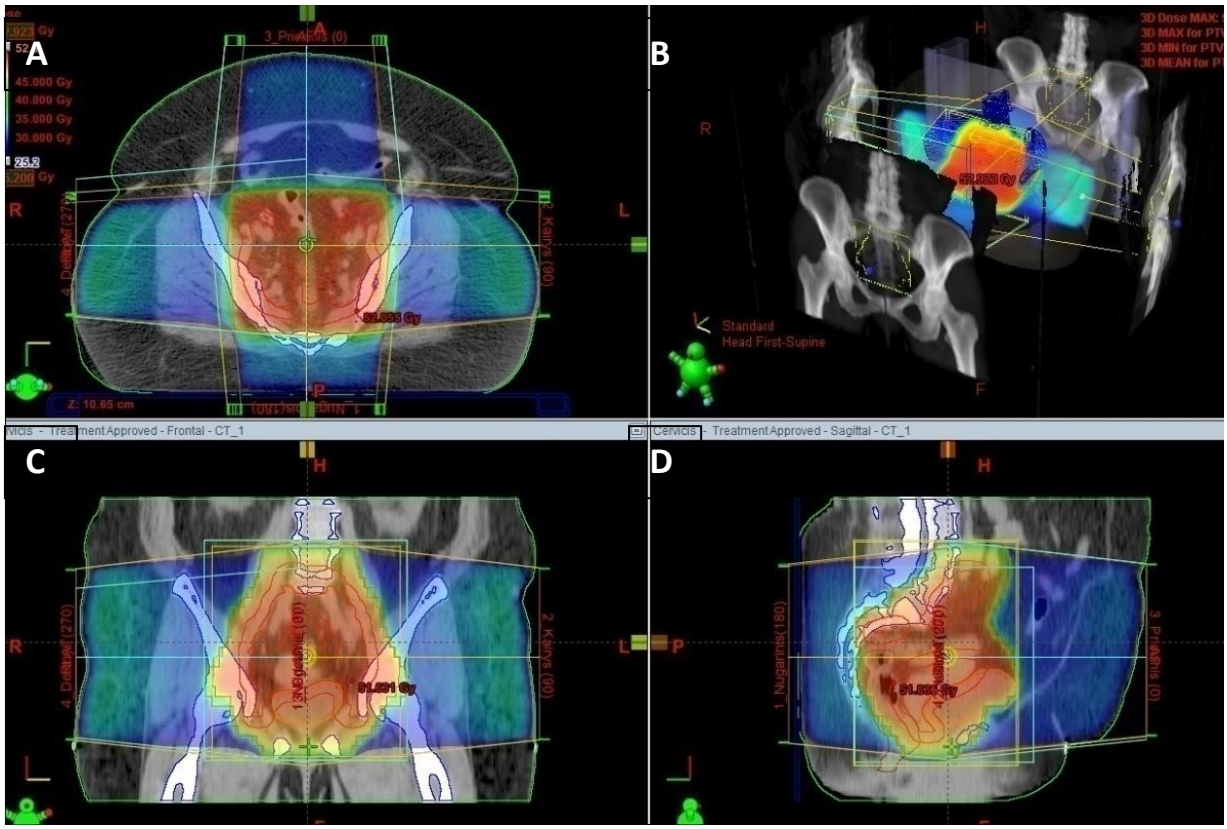


Figure 2.2.3.2. A plan of three-dimensional conformal external beam radiotherapy (EBRT) for cervical cancer. Distribution of ionized radiation administered in axial section of computed tomography (A), tree-dimensional image (B), coronary reconstruction (C) and sagittal reconstruction (D) is shown.

Chemoradiotherapy. Chemoradiotherapy started in week 6. A combination of cisplatin (40 mg/m²) and gemcitabine (125 mg/m²) was administered weekly for 5 consecutive weeks during EBRT, beginning on the first day of radiation. The infusion of cytostatics was administered prior to the procedure of the radiotherapy.

The radiotherapy was targeted on the cervix, superior third of the vagina, uterus, parametrium and regional lymph nodes (including common iliac, obturator, pre-sacral ones). EBRT was implemented using linear energy accelerator: energy 15 MeV, average dose (AD) 1.8–2.0 Gy, 25–28 fractions 5 days a week; total dose (TD) – 50–50.4 Gy. Intracavitary brachytherapy under general anaesthesia was given following the completion of EBRT. Patients received 4 fractions (each 7 Gy) to point A high dose rate (HDR) 1–2 per week. The total dose delivered to point A was 89 Gy.

The response to the treatment was evaluated 2 months after the completion of the treatment. The patients underwent control vaginal and rectovaginal examinations, oncocytology test (in event of suspicion of remaining tumour biopsy was performed) and control radiology investigations, in order to evaluate the efficacy of the treatment. Later on, the patients were observed in accordance with standard programme of observation after the treatment of cervical cancer. When the progression of the disease had been diagnosed, standard treatment for progression of cervical cancer was applied.

2.2.4. Assessment of efficacy of the treatment

Evaluation of the response to the neoadjuvant chemotherapy was based on pelvic examination (vaginal and rectovaginal examination), control standard and perfusion CT; the latter was used for assessment of the size and structure of the cervix, condition of parametrium and regional lymph nodes after the neoadjuvant chemotherapy. It was supposed that complete response was characterized by full disappearance of tumour signs and partial response was characterized by a decrease of these signs. In event of unchanged tumour size, stabilization of the illness was stated, while the increase of tumour size for > 20% or development of new lesions (metastases) was regarded to be progression of the disease.

The response to the complete study Protocol planned treatment was assessed by means of pelvic examination and oncocytology test (in event of suspicion of residual tumour – on morphology examination of biopsy). The complete response was stated when no clinical or cytology signs of the tumour were present. The response was evaluated using control standard and perfusion CT of the cervix. In order to exclude distant metastases (progression of the disease), control radiological investigations (X-ray, ultrasound examination and CT) of the chest, abdomen and pelvis were performed.

The duration of the period without disease progression (time to progression) was measured from the first day of the treatment according to the study protocol to the date on which disease progression was detected. Overall survival was measured from the first day of the treatment to the date on which the patient dies from any cause.

Adverse events of the treatment were assessed in accordance with criteria of Common Terminology Criteria for Averse Events (version 3.0); toxicity of the radiotherapy was evaluated using Radiation Therapy Oncology Group/European

Organisation for Research and Treatment of cancer criteria for assessment of toxic effect of radiation therapy. The symptom was evaluated using grading system from 0 (no symptoms were present) to 4 (very severe symptoms).

2.3. Laboratory section of the study

Blood samples for GSH, GST and *GSTM1*, *GSTT1* genes polymorphism examination of patients with cervical cancer were obtained prior treatment, 2 weeks after the neoadjuvant therapy (before initiation of the chemoradiotherapy) and 2 months after the completion of the chemoradiotherapy. The blood samples of women with cervical intraepithelial lesions, as well as these of healthy women were taken only once. The blood samples from women with cervical intraepithelial lesions were obtained prior conisation.

2.3.1. Assessment of GSH and GST

The blood for GSH and GST assessment was centrifuged at the rate of 3000 rev/min for 30 min. Blood serum was stored in a microtube at temperature of -70°C before examination. Serum GSH and GST content were assessed by means of ELISA method (CUSABIO, BIOTECH, China), following the guidelines of the manufacturer. The methods of operation of both kits are similar; therefore, they are discussed together.

Before the reaction, all the reagents used in the kit were warmed up to room temperature. The kit contained microplates with wells coated with monoclonal antibodies, specific to human GSH and GSTpi. All the control and standard samples were diluted using dilution buffer to the level indicated.

Incubation buffer solution of 100 μl was added to each well; then 100 μl of standard (i.e. of known GSH and GST level) and examined solution were added. After that, the microplate was covered with an adhesive strip and incubated at 37°C for 2 hours. The liquid was removed from the wells after incubation. Biotin antibody working solution 100 μl was added to each well and incubated for 1 hour at 37°C. The liquid was evacuated from the wells (the plate was washed 3 times using wash buffer, adding 200 μl of the buffer into each well) after incubation. Then 100 μl of HRP avidin working solution was added into each well, the microplates were covered with a new adhesive

strip and incubated for 1 hour at 37°C. After incubation, the liquid was evacuated from the wells and the plate was washed for 5 times using wash buffer (adding 200 μl into each well). Then 90 μl of prepared tetramethylbenzidine substrate solution was added into each well and incubated in the dark for 10 – 30 min. at 37°C. The reaction was stopped by adding 50 μl of buffer solution into each well.

Absorption was measured by means of spectrophotometry, using microplate reader (*Shenzhen Mindray Bio-Medical Electronics Co*, China), set to wavelength of 450 nm. The levels of the samples were calculated using the standard curves.

2.3.2. Assessment of glutathione S-transferase *GSTM1* and *GSTT1* genes polymorphism

The 1st stage. DNA purification from blood. In order to purify genomic DNA from blood, *GeneJE Genomic DNA purification Kit* (*Thermo Fisher*, USA) was used. The purification was performed in accordance with manufacturer's guidelines.

At first, 200 μl of blood were added to 400 μl of lysis buffer solution and 20 μl of proteinase K solution. The mixture was incubated and continuously shaken for 10 min. at 56^{o}C. After that, 200 μl of 96% of ethanol was added and mixed. The resulting lysate was added into spin column and centrifuged for 1 min. at 6000 x g. Afterwards, the column was placed into a new microtube. After adding of 500 μ of Wash Buffer I solution, centrifugation at 8000 x g was performed for 1 min. Then 500 μl of Wash Buffer II was added into the column and centrifugation at maximum rate performed for 3 min. Then the column was placed into a new microtube. After addition of 200 μl of elution buffer solution, the column was incubated at room temperature for 2 min. Centrifugation at 8000 x g for 1 min. was performed. After extraction of the column, purified DNA remained in the microtube. The concentration of DNA was measured using *Nanodrop 2000* spectrophotometer (*Thermo Fisher*, USA).

The 2nd stage. Multiplex polymerase chain reaction. In order to identify *GSTM1* and *GSTT1* genes polymorphism, multiplex polymerase chain reaction (PCR) was performed. The reaction mixture contained 5 μl DNA, 5 μl 10xCPR buffer solution, 2 μl forward *GSTM1* primer, 2 μl reverse *GSTM1* primer, 2 μl forward *GSTT1* primer, 2 μl reverse *GSTT1* primer, 0.5 μl Taq DNA polymerase and water to 50 μl. The polymerase

chain reaction was performed in following way: initial step – denaturation for 2 min. at 95°C; 30 similar cycles: denaturation for 1 min. at 94°C; annealing of the primer step for 1 min. at 61°C; elongation of DNA chain step for 1 min. at 72°C; final cycle – elongation for 5 min. at 72°C. The products obtained using PCR were analysed in agarose gel and compared with DNA standard of length. The photos of the results were taken in UV light (Fig. 2.3.2.1).

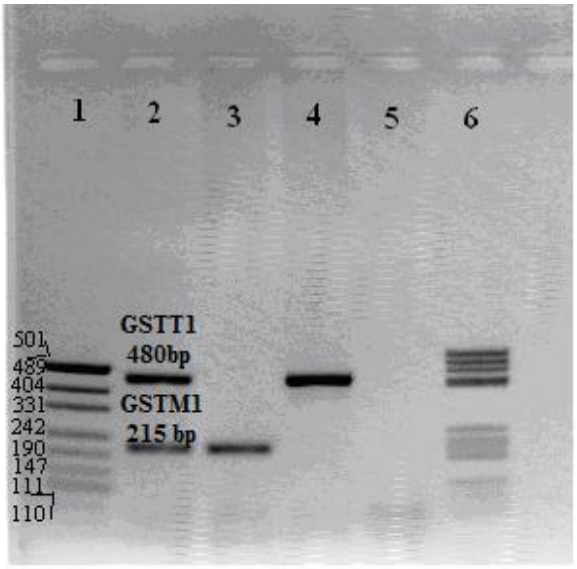


Figure 2.3.2.1. Analysis of *GSTM1* and *GSTT1* genes polymorphism by means of multiplex polymerase chain reaction

The 1st lane presents DNA length standard *pUCMixMarker 19*, 2nd lane shows wild type of *GSTT1* and *GSTM1*; in 3rd lane *GSTT1* deletion and *GSTM1* wild type are present; 4th lane demonstrates *GSTT1* wild type and *GSTM1* deletion; 5th lane presents deletion of *GSTT1* and *GSTM1* and in the 6th lane DNA length standard *pBR322 DNA/ BsuRI (Hae III*) is presented.

2.4. Statistical data analysis

In order to evaluate qualitative variables, frequency of variables (n) and relative frequency (%) were calculated. Data distribution normality was checked using the Shapiro-Wilk test. Because the data did not meet the normal distribution, non-parametric methods were used. Chi-square (χ^2) association criterion was used to check interrelationship of qualitative variables. As the test sample was small, the comparison

between groups uses statistical tests, which are recommended in small samples. For quantitative variables, pair-wise comparisons were conducted using the Wilcoxon signed rank test for dependent samples. Mann-Whitney test was used to compare means of two independent samples. The results were considered to be statistically significant, when $p<0.05$. Survival was analysed by means of Kaplan-Meier method. The survival curves were compared using log-rank test. The analysis of data was performed using software package for data analysis IBM *SPSS 21.0* (*Armonk, NY: IBM Corp*, USA, 2012).

3. RESULTS

3.1. Clinical section

The clinical study included 36 patients with locally advanced FIGO stage IIB-IIIB cervical cancer. During this perspective study, the efficacy and safety (including response to the treatment, time to disease progression and overall survival rate of the patients) of new intense neoadjuvant chemotherapy followed by chemoradiotherapy were evaluated. The results of efficacy of the treatment were compared between the patients in accordance with the stage of cervical cancer.

3.1.1. Treatment of the patients

The data regarding implementation of the Protocol-planned treatment of patients with locally advanced cervical cancer are presented in Table 3.1.1.1. Neoadjuvant chemotherapy was completely performed for 97.2% of the patients; chemotherapy during the period of EBRT – for 27.7% of the patients. The EBRT was completed for 91.7% and brachytherapy for 88.9% of the patients.

Table 3.1.1.1. Implementation of the Protocol-planned treatment

Treatment	n (%)
Neoadjuvant chemotherapy was implemented:	
partially (3 infusions)	1 (2.8)
completely (4 infusions)	35 (97.2)
Chemotherapy during radiotherapy was implemented:	
partially (2 infusions)	2 (5.6)
partially (3 infusions)	9 (25)
partially (4 infusions)	15 (41.7)
completely (5 infusions)	10 (27.7)
The dose of external beam radiotherapy was implemented:	
partially	3 (8.3)
completely	33 (91.7)
Brachytherapy was implemented:	
partially	4 (11.1)
completely	32 (88.9)

n – number of patients.

3.1.2. Efficacy of the treatment

Neoadjuvant chemotherapy. The effect of the treatment was evaluated in 36 patients (Tab. 3.1.2.1). The evaluation was based on clinical examination and radiological investigations and showed that the rate of response was 72.2% (n=26; 95% CI 64.76 – 79.69). Partial response was stated in all of these patients. Stable course of the disease was stated in 27.8% of the patients (n=10; 95% CI 20.31 – 35.24). There were no cases of progression of the disease after the neoadjuvant chemotherapy.

Table 3.1.2.1. Response to the neoadjuvant chemotherapy in patients with locally advanced cervical cancer

Type of response	**Response to the treatment**							
	Patients		Distribution of the patients according to the stage					
			II B stage		III A stage		III B stage	
	n	% (95% CI)	n	% (95% CI)	n	% (95% CI)	n	% (95% CI)
Partial response	26	72.2 (64.76–79.69)	12	70.6 (59.54–81.64)	1	100	13	72.2 (61.67–82.78)
Stable course	10	27.8 (20.31–35.24)	5	29.4 (18.36–40.46)	–	–	5	27.8 (17.22–38.33)

CI – confidence interval; n – number of patients.

Chemoradiotherapy. The effect of the treatment was evaluated in 34 patients. Two patients missed visit scheduled in the Protocol because of personal reasons, not related to the study. Clinical and cytological response was present in 94.1% of the patients (n=32; 95% CI 90.08–98.15) and 5.9% of the patients (n=2; 95% CI 1.85–9.92) had progression of the disease after the treatment. In one patient progression of the disease involved lungs and inguinal lymph nodes, in another the progression involved bones (Tab.3.1.2.2).

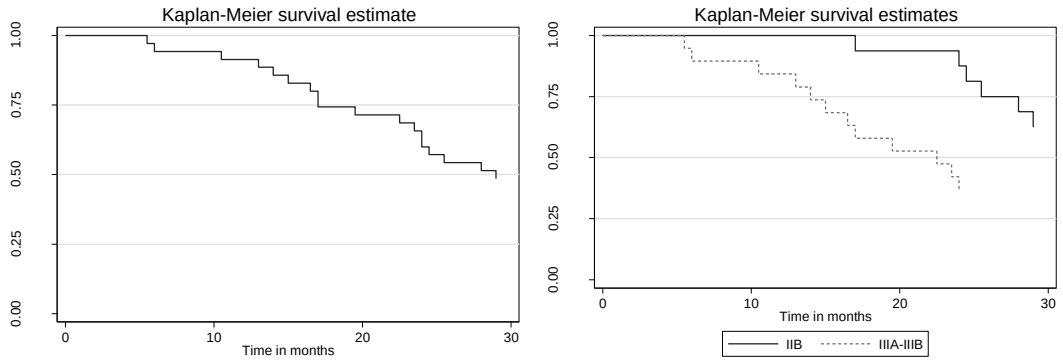
Table 3.1.2.2. Response to the complete treatment in patients with locally advanced cervical cancer

Type of response	**Response to the treatment**							
	Patients		Distribution of the patients according to the stage					
			II B stage		III A stage		III B stage	
	n	% (95% CI)	n	% (95% CI)	n	% (95% CI)	n	% (95% CI)
Complete response	32	94.1 (90.08–98.15)	15	93.8 (87.70–99.80)	1	100	16	94.1 (88.41–99.82)
Progression of the disease	2	5.9 (1.85–9.92)	1	6.3 (0.20–12.30)	–	–	1	5.9 (0.18–11.59)

CI - confidence interval; n – number of patients.

The time to disease progression was evaluated (analysis of data was performed on the 1st of November, 2014). Mean duration of observation of the patients was 36 months (ranging from 24.5 to 54.5). Data of 35 patients were assessed, as one of the patients did not attend the Clinic of National Cancer Institute (emigrated). At the time of data analysis, 71.4% (n=25; 95% CI 63.79 – 79.6) patients were without disease progression and 28.6% (n=10; 95% CI 20.94 – 36.21) had progression of the disease. During the first year after initiation of the treatment, progression of the disease was diagnosed for 8.6% (n=3; 95% CI 5.80 – 11.40) of the patients; for two of them progression was diagnosed just after completion of the treatment. A local pelvic progression was diagnosed for one patient, one patient developed bone metastases and the last one experienced metastases in lungs and inguinal lymph nodes. Two years after the treatment, the progression of the disease was diagnosed for 28.6% (n=10; 95% CI 24.08 – 33.12) of the patients, including those who had progression during the first year (Fig.4.1.2.1). Lymph nodes metastases were detected in 14.3% (n=5), local progression in pelvis in 11.4% (n=4), lung metastases in 8.6% (n=3), metastases in bones in 5.7% (n=2), in liver 2.9% (n=1) of the cases. Progression-free survival was assessed using Kaplan-Meier method (Fig.3.1.2.1).

A B



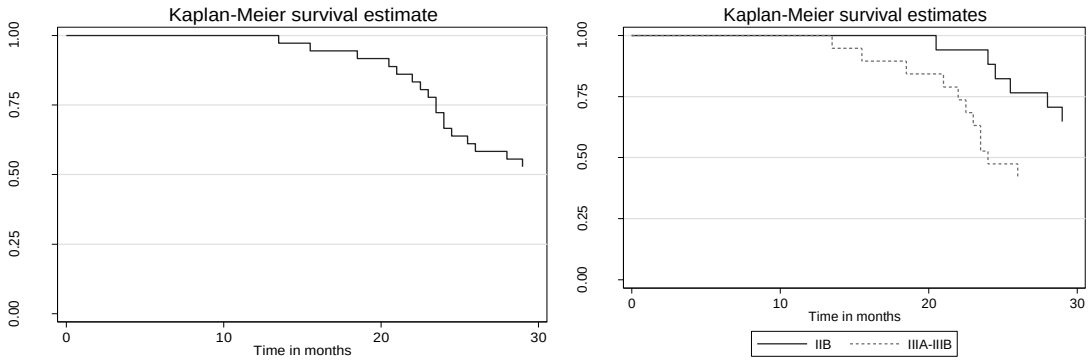
p = 0.0091

Figure 3.1.2.1. Progression-free survival of patients with cervical cancer: A – progression-free survival of all study patients; B – progression-free survival of patients depending on the stage of the disease

The results of progression-free survival of the patients were assessed in accordance with the stage of the disease. Data presented in Fig. 3.1.2.1 showed that in patients for whom stage IIB of cervical cancer was diagnosed, the results of progression-free survival after the neoadjuvant chemotherapy followed by chemoradiotherapy were statistically significantly better, than these of patients with stage III cervical cancer.

To the date of analysis of the study data, 75% (n=27; 95% CI 70.67 – 79.33) of the patients were alive and 25% (n=9; 95% CI 20.67 – 29.33) had died. All the patients (n=35) survived for at least one year, 27 patients (77.1%; 95% CI 72.90 – 81.30) had survived for two years. The evaluation of overall survival in accordance with the stage of the disease showed that 94.1% (n=16; 95% CI 91.74 – 96.46) of the patients with stage IIB cervical cancer survived for 2 years, whereas 2-year survival rate among the patients suffering from stage III cervical cancer was 57.8% (n=11; 95% CI 52.86 – 62.74); statistically significant difference in overall survival was found out between these groups (Fig. 3.1.2.2).

A B



p = 0.0229

Figure 3.1.2.2. Overall survival of the patients with cervical cancer: A - overall survival of the all study patients; B – overall survival of the patients depending on the stage of the disease

The observation of the patients who were treated in our prospective clinical study is going on. We expect to compare the results of 5-year survival of patients who were treated by means of the intense (weekly) cisplatin and gemcitabine neoadjuvant therapy followed by chemoradiotherapy with the results of treatment of locally advanced cervical cancer obtained by other authors.

3.1.3. Safety of the treatment

The neoadjuvant therapy was completely implemented for 97.2% of the patients. For one patient 1 infusion was not performed because of grade III leukopenia. The following toxic effect of the neoadjuvant chemotherapy on haemopoiesis of the patients was revealed: grade II anaemia - 22% (n=8), grade II leukopenia - 11% (n=4); grade II neutropenia - 25% (n=9); grade III neutropenia - 8% (n=3).

For 69.4% of the patients $\geq$ 4 infusions of cytostatic drugs were performed on chemoradiotherapy. The toxicity of the treatment affected haemopoiesis, digestive tract

and urinary system most commonly: grade III anaemia was diagnosed for 11% (n=4), leukopenia for 47% (n=17), neutropenia for 42% (n=15), thrombocytopenia for 17% (n=6), diarrhoea for 8% (n=3) of the patients; grade IV leukopenia was present in 3% (n=1), neutropenia in 5% (n=2) and thrombocytopenia in 3% (n=1) of the patients. Another common grade III or IV toxic effect was electrolyte imbalance related to diarrhoea; it was present in 12% (n=5) and 11 (n=4) of the patients, respectively. The cases of low grade asthenia, increase of alanine aminotransferase (ALT), aspartate aminotransferase (AST) were observed, also. Table 3.1.3.1 presents data of toxic effect in details.

Table 3.1.3.1. Acute toxicity effects in the study patients with locally advanced cervical cancer

Toxic effects	**Neoadjuvant chemotherapy**				**Chemoradiotherapy**			
	Iº n (%)	IIº n (%)	IIIº n (%)	IVº n (%)	Iº n (%)	IIº n (%)	IIIº n (%)	IVº n (%)
Haemopoiesis:								
Anaemia	16 (44.4)	8 (22.2)	-	-	13 (36.1)	19 (52.8)	4 (11.1)	-
Leukopenia	13 (36.1)	4 (11.1)	-	-	3 (8.3)	15 (41.7)	17 (47.2)	1 (2.8)
Neutropenia	10 (27.8)	9 (25.0)	3 (8.3)	-	8 (22.2)	11 (30.5)	15 (41.7)	2 (5.5)
Thrombocyto-penia	23 (63.9)	-	-	-	16 (44.4)	13 (36.1)	6 (16.7)	1 (2.8)
Other systems:								
Diarrhoea	-	-	-	-	13 (36.1)	14 (38.9)	3 (8.3)	-
Cystitis	-	-	-	-	16 (44.4)	9 (25.0)	-	-
Nausea	8 (22.2)	-	-	-	6 (16.7)	4 (11.1)	-	-
Changes of skin/mucosa	-	-	-	-	2 (5.5)	3 (8.3)	-	-
Asthenia	2 (5.5)	-	-	-	19 (52.8)	4 (11.1)	-	-
Other	7 (19.4)	-	-	-	33 (91.7)	9 (25.0)	5 (13.9)	4 (11.1)

n – number of cases.

The following late adverse reactions were observed among the patients: radiation cystitis – 19% (n=7), radiation proctitis – 8% (n=3), hydronephrosis caused by post-radiation fibrosis – 11% (n=4), and adhesive ileus – 6% (n=2). The observation of the patients is going on.

3.2. Laboratory section of the study

3.2.1. Blood serum levels of reduced glutathione (GSH) and glutathione S-transferase (GST) in patients with cervical cancer, women with cervical intraepithelial lesions and healthy women

Blood serum GSH and GST levels were assessed for patients with locally advanced cervical cancer (before the treatment, after the neoadjuvant therapy (NChT) and after the chemoradiotherapy (ChRT), women with cervical intraepithelial lesions (CIN3/CIS) and healthy women. The data are presented in Table 3.2.1.1.

Table 3.2.1.1. Blood serum levels of reduced glutathione (GSH) and glutathione S-transferase (GST) of the study subjects

Group		**GSH, µg/ml ± SD**	**GST, ng/ml ± SD**
Patients with cervical cancer (n=36)	Before treatment	23.5 ± 13.97	0.58 ± 1.11
	After NChT	35.3 ± 24.83	0.76 ± 1.67
	After ChRT*	32.0 ± 25.12	0.63 ± 0.90
Women with CIN3/CIS (n=27)		69.7 ± 19.77	0.26 ± 0.15
Healthy women (n=31)		55.4 ± 20.14	0.48 ± 0.23

SD – standard deviation; n – number of cases; CIN3/CIS – cervical intraepithelial neoplasia grade 3/carcinoma *in situ*; NChT – neoadjuvant chemotherapy; ChRT – chemoradiotherapy; * – data of 34 patients were evaluated, as 2 patients had not attended the study at scheduled time.

The highest GSH level was detected in women with cervical intraepithelial lesions and the lowest one – in patients with cervical cancer; the highest GST level was found in patients with cervical cancer and the lowest one – in women with cervical intraepithelial lesions.

The comparison of GSH and GST levels in patients with cervical cancer (before the treatment), women with cervical intraepithelial lesions (before the conisation of the cervix) and healthy women demonstrated statistically significant differences (p=0.0001 and p=0.0008, respectively).

The analysis of GST and GSH blood serum levels in separate groups showed that in patients with locally advanced cervical cancer the level of GSH was significantly lower (p=0.0001), in comparison with this of women with cervical intraepithelial lesions or healthy women; on the other hand, in patients with cervical cancer the level of GST was highest and statistically significantly differed (p=0.0095) from this of healthy

women. It should be noted, that GST level in patients with cervical cancer was higher than level detected in women with cervical intraepithelial lesions; however, there were no statistically significant difference between these groups. The level of GSH in women with cervical intraepithelial lesions was statistically significantly higher (p=0.0006) and GST level – lower (p=0.001), in comparison with these in the blood serum of healthy women (Table 3.2.1.1).

3.2.2. Changes of reduced glutathione (GSH) and glutathione S-transferase (GST) levels during the process of treatment

The examination of GSH and GST blood serum levels of patients with stage IIB – IIIB cervical cancer, in accordance with treatment applied, demonstrated statistically significant ($p<0.05$) increase of GSH after the neoadjuvant chemotherapy. The level of GSH remained the same as after the neoadjuvant chemotherapy, when subsequent chemoradiotherapy was performed. No statistically significant changes of GST level were revealed during the process of the treatment (Tab.3.2.2.1).

Table 3.2.2.1. Changes of reduced glutathione (GSH) and glutathione S-transferase (GST) levels in patients with cervical cancer during the process of the treatment

Factor	**n**	**Before treatment (mean±SD)**	**n**	**After neoadjuvant chemotherapy (mean±SD)**	**n**	**After chemoradiotherapy (mean±SD)**
GSH, µg/ml	36	23.51±13.97*	36	35.29±24.83*	34	32.01±25.12
GST, ng/ml	36	0.58±1.11	36	0.76±1.67	34	0.63±0.90

* $p<0.05$.

After distribution of the patients, according to the stage of the disease, it became obvious that GSH level statistically significantly increased in event of both IIB and IIIB

stages after the neoadjuvant chemotherapy; in event of IIB stage the level of GSH was statistically significantly lower after the chemoradiotherapy, in comparison with the level after the neoadjuvant chemotherapy. Statistically significant changes of GST level during the process of treatment were found neither in patients with IIB, nor in patients with IIIB stage of cervical cancer. However, the comparison of these groups after the chemoradiotherapy showed a statistically significant difference: the level of GST was higher in blood serum of patients with IIB stage of cervical cancer (Tab. 3.2.2.2).

Table 3.2.2.2. Changes of reduced glutathione (GSH) and glutathione S-transferase (GST) blood serum levels during the process of treatment of patients with cervical cancer, in accordance with the stage of the disease

FIGO-II B						
Factor	**n**	**Before treatment (mean±SD)**	**n**	**After neoadjuvant chemotherapy (mean±SD)**	**n**	**After chemoradiotherapy (mean±SN)**
GSH, µg/ml	17	23.51±13.97∗	17	37.84±28.63∗●	15	31.60±21.11●
GST, ng/ml	17	0.79±1.41	17	0.78±1.50	15	0.91±1.08○
FIGO-III B						
GSH, µg/ml	19	22.72±13.88∗	19	33.01±21.42∗	19	29.92±27.43
GST, ng/ml	19	0.40±0.74	19	0.74±1.85	19	0.39±0.59○

∗, ●, ○ $p<0.05$.

The study patients were distributed into the groups, in accordance with regional lymph nodes metastases found on radiological examinations; in event of absence of metastases in regional lymph nodes (N0), there was statistically significant decrease of GSH level after the chemoradiotherapy, in comparison with that after the neoadjuvant chemotherapy; when regional lymph nodes metastases were diagnosed (N1), GSH level

statistically significantly increased after the neoadjuvant therapy and remained statistically significantly increased after the chemoradiotherapy, as well. During the process of the treatment, statistically significant changes of GST level, according to condition of regional lymph nodes, were not present: the increase of GST level after the chemoradiotherapy in patients without lymph nodes metastases and, after the neoadjuvant therapy, in patients with lymph nodes metastases, was statistically insignificant (Tab. 3.2.2.3).

Table 3.2.2.3. Changes of reduced glutathione (GSH) and glutathione S-transferase (GST) levels during the treatment period in patients with cervical cancer, in accordance with regional lymph nodes metastases detected using radiological examination

N0						
Factor	**n**	**Before treatment (mean±SD)**	**n**	**After neoadjuvant chemotherapy (mean±SD)**	**n**	**After chemoradiotherapy (mean±SD)**
GSH, µg/ml	15	26.70±14.17	15	36.46±28.51●	13	26.77±21.43●
GST, ng/ml	15	0.45±0.37	15	0.38±0.30	13	0.84±1.19
N1						
GSH, µg/ml	21	21.23±13.72*●	21	34.46±22.54*	21	33.07±26.44●
GST, ng/ml	21	0.68±1.42	21	1.03±2.15	21	0.48±0.58

*, ● $p<0.05$.

The patients were distributed into two groups, in order to evaluate changes of GSH and GST levels during the whole period of treatment, in accordance with response to the neoadjuvant chemotherapy by means of cisplatin and gemcitabine combination: the first group (n=26) included patients with positive (in all patients partial) response to the treatment, the second group (n=10) included patients with stable course of the disease. It should be noted, that in event of positive response, the level of GSH increased

statistically significantly after the neoadjuvant chemotherapy. The results of examination of variables mentioned above in patients of this group after the chemoradiotherapy showed statistically significant decrease of GSH level, in comparison with that detected after the neoadjuvant therapy. During the period of treatment, no statistically significant changes of GSH or GST were detected in patients not responding to the neoadjuvant chemotherapy (Tab. 3.2.2.4).

Table 3.2.2.4. Changes of reduced glutathione (GSH) and glutathione S-transferase (GST) levels during the period of treatment, in accordance with response to the neoadjuvant chemotherapy

Factor	**n**	**Before treatment (mean±SD)**	**n**	**After neoadjuvant chemotherapy (mean±SD)**	**n**	**After chemoradiotherapy (mean±SD)**
Positive response to neoadjuvant chemotherapy						
GSH, µg/ml	26	25.08±14.83*	26	38.39±23.60*●	26	33.21±26.19●
GST, ng/ml	26	0.48±0.67	26	0.66±1.58	26	0.56±0.92
No response to neoadjuvant chemotherapy						
GSH, µg/ml	10	19.41±11.05	10	27.24±27.41	10	24.55±19.74
GST, ng/ml	10	0.85±1.85	10	1.01±1.95	10	0.77±0.78

*, ● $p<0.05$.

After completion of the complete treatment, 2 patients had not attended the visits for evaluation of the treatment efficacy, in 2 patients progression of the disease was diagnosed and 32 patients showed complete response to the treatment. The evaluation of GSH and GST levels during the period of treatment of the latter group of patients showed statistically significant increase of GSH after the neoadjuvant chemotherapy and statistically significant decrease of this variable after the chemoradiotherapy (Tab. 3.2.2.5).

Table 3.2.2.5. Changes of reduced glutathione (GSH) and glutathione S-transferase (GST) levels, in accordance with complete response to chemoradiotherapy

Factor	**n**	**Before treatment (mean±SD)**	**n**	**After neoadjuvant chemotherapy (mean±SD)**	**n**	**After chemoradiotherapy (mean±SD)**
GSH, μg/ml	32	23.42±14.38∗	32	35.62±25.01∗●	32	32.01±25.12●
GST, ng/ml	32	0.62±1.19	32	0.56±1.15	32	0.63±0.90

∗, ● $p<0.05$.

The comparison of GSH and GST blood serum levels in patients with cervical cancer after chemoradiotherapy, women with cervical intraepithelial lesions and healthy women demonstrated statistically significant differences (p=0.0001 and p=0.0026, repectively). It should be noted that two months after the treatment, GSH and GST blood serum levels in patients with cervical cancer differed from these of healthy women, i.e. did not achieved the levels found in healthy women. The levels of GSH and GST in patients with cervical cancer differed significantly from these of healthy women, both after the neoadjuvant chemotherapy and after the complete chemoradiotherapy (GSH μg/ml, p=0.0003 and p=0.0001, respectively; GST ng/ml, p=0.011 and p=0.088, respectively) (Table 3.2.1.1).

The observation of the patients after the treatment was continued and progression-free survival was evaluated. During the first two years after initiation of the treatment, the disease progressed in 28.6% of patients (n=10), including 2 patients in whom progression of the disease was diagnosed just after the chemoradiotherapy. The data regarding changes of GSH and GST levels, in accordance with progression of cervical cancer during the first two years after the initiation of the treatment, are presented in Table 3.2.2.6. Statistically significant increase of GSH level after neoadjuvant chemotherapy and statistically significant decrease of this level after chemoradiotherapy were found in patients without disease progression. In patients who experienced progression of the

disease during the first two years after the treatment, statistically significant changes of GSH level were not present during the process of treatment. On one hand, no statistically significant changes of GST during the process of treatment were found out, on other hand, the comparison of GST level after the chemoradiotherapy showed statistically significant differences of GST levels in patients without disease progression and patients with progression of the disease (Tab.3.2.2.6).

Table 3.2.2.6. Changes of reduced glutathione (GSH) and glutathione S-transferase (GST) levels in patients with cervical cancer, in accordance with progression of the disease during two years after the treatment

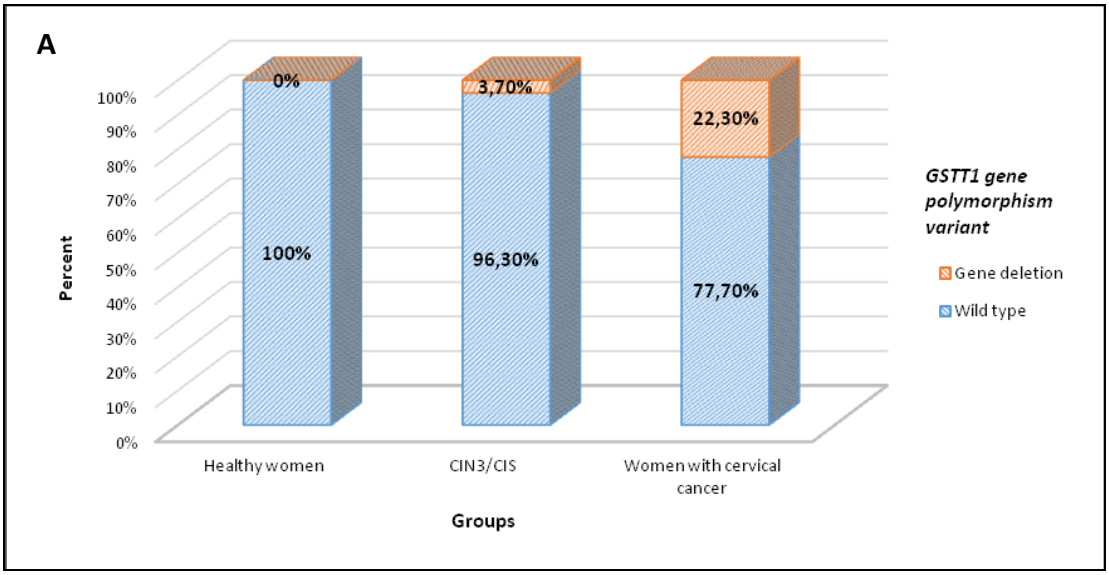
Factor	**n**	**Before treatment (mean±SD)**	**n**	**After neoadjuvant chemotherapy (mean±SD)**	**n°**	**After chemoradiotherapy (mean±SD)**
Progression-free						
GSH, µg/ml	25	24.20±15.44*	25	39.07±27.02*●	25	34.62±27.15●
GST, ng/ml	25	0.39±0.31	25	0.37±0.35	25	0.74±1.00○
Disease progression						
GSH, µg/ml	10	21.70±9.61	10	25.46±14.90	10	21.17±13.22
GST, ng/ml	10	1.10±2.03	10	1.77±2.99	10	0.33±0.32○

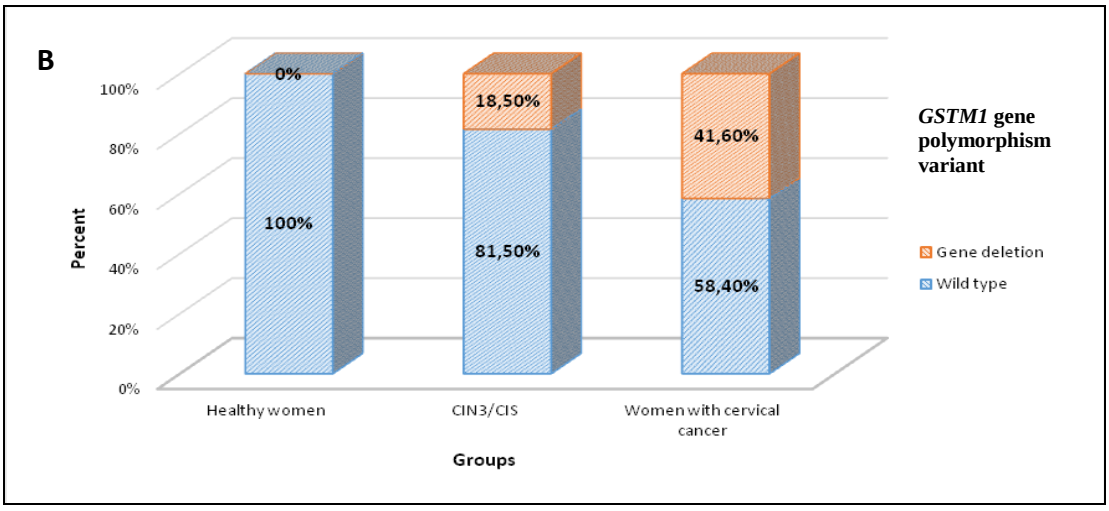
*, ●, ○ $p<0.05$.

3.2.3. Investigations of glutathione S-transferase *GSTM1* and *GSTT1* genes polymorphism

The investigation of *GSTM1* and *GSTT1* genes polymorphism in 31 healthy women, showed no cases of deletion in these genes. In 27 women with cervical intraepithelial lesions (CIN3/CIS), deletion of *GSTM1* was identified for 18.5% (n=5) of the patients; however, deletion of *GSTT1* was identified for 3.7% (n=1) of the patients with locally advanced cervical cancer. It should be noted that among 36 patients with

cervical cancer, deletion of *GSTM1* gene was present in 41.6 % (n=15) and *GSTM1* gene deletion in 22.3 % (n=8) of the patients. Statistically significant difference of distribution between all groups studied was found out ($p<0.01$ for *GSTM1* and $p=0.004$ for *GSTT1*) (Fig. 3.2.3.1).





CIN3/ CIS – cervical intraepithelial neoplasia grade 3/carcinoma *in situ.*

Fig. 3.2.3.1. Distribution of glutathione S-transferase genes polymorphism between the study groups: A – *GSTT1*, B – *GSTM1*

Further comparison of *GSTM1* and *GSTT1* genes polymorphism distribution among healthy women and women with cervical intraepithelial lesions was performed. Statistically significant distribution of *GSTM1* gene polymorphism among healthy women and women with cervical intraepithelial lesions was revealed; however, for *GSTT1* gene polymorphism, there was no statistically significant difference between these groups (Tab. 3.2.3.1).

Table 3.2.3.1. Distribution of glutathione S-transferase *GSTM1* and *GSTT1* genes polymorphism variants between healthy women and women with cervical intraepithelial lesions

Polymorphism variant	***GSTM1***		***GSTT*1**	
	Healthy women, n	**Women with CIN3/CIS, n**	**Healthy women, n**	**Women with CIN3/CIS, n**
Deletion	0	5	0	1
Wild type	31	22	31	26
p	0.01		0.46	

n – number of cases, CIN3/CIS – intraepithelial cervical neoplasia grade 3/carcinoma *in situ.*

The comparison of healthy women and women with cervical cancer showed statistically significant difference between the groups, both for *GSTM1* and *GSTT1* genes polymorphism (Tab. 3.2.3.2).

Table 3.2.3.2. Distribution of glutathione S-transferase *GSTM1* and *GSTT1* genes polymorphism variants between healthy women and women with cervical cancer

Polymorphism variant	***GSTM1***		***GSTT1***	
	Healthy women, n	**Women with cervical cancer, n**	**Healthy women, n**	**Women with cervical cancer, n**
Deletion	0	15	0	8
Wild type	31	21	31	28
p	0.001		0.005	

n – number of cases.

Statistically significant difference of distribution of *GSTM1* and *GSTT1* genes polymorphism variants was found between the women with cervical intraepithelial lesions and women with cervical cancer (Tab.3.2.3.3).

Table 3.2.3.3. Distribution of glutathione S-transferase *GSTM1* and *GSTT1* genes polymorphism variants between women with cervical intraepithelial lesions and women with cervical cancer

Polymorphism variant	***GSTM1***		***GSTT1***	
	CIN3/ CIS diagnosed, n	**Women with cervical cancer, n**	**CIN3/ CIS diagnosed, n**	**Women with cervical cancer, n**
Deletion	5	15	1	8
Wild type	22	21	26	28
p	0.04		0.03	

n – number of cases, CIN3/CIS – cervical intraepithelial neoplasia grade 3/carcinoma *in situ.*

Distribution of *GSTM1* and *GSTT1* genes polymorphism in women with cervical cancer was also evaluated, in accordance with the disease stage, tumour histological type,

tumour differentiation grade, regional lymph nodes status, response to the treatment and progression of the disease during the first two years after initiation of the treatment. The results of investigations obtained did not demonstrate statistically significant relationship.

4. CONCLUSIONS

1. Statistically significant differences in blood serum levels of reduced glutathione (GSH) and glutathione S-transferase (GST) were detected in patients with locally advanced cervical cancer, women with cervical intraepithelial lesions (CIN3/CIS) and healthy women.
2. Deletions of glutathione S-transferase *GSTM1* and *GSTT1* genes were not detected in healthy women. The incidence of genes deletions was not significant in women with cervical intraepithelial lesions (CIN3/CIS). The highest incidence of genes deletions was found in patients with locally advanced cervical cancer.
3. The treatment of patients with locally advanced stage IIB-IIIB cancer demonstrated that:
    - short and intense weekly course of neoadjuvant chemotherapy by means of cisplatin and gemcitabine before chemoradiation treatment was effective and safe;
    - chemoradiotherapy (using the same combination of cytostatic drugs), performed after neoadjuvant therapy, was effective; however, side effects of the treatment on haemopoiesis limited the implementation of the complete treatment.
4. The investigation of changes in reduced glutathione (GSH) and glutathione S-transferase (GST) levels during the process of the cervical cancer treatment demonstrated that:
    - statistically significant changes of GSH level were observed depending on the cervical cancer stage, the regional lymph nodes status , as well as on the positive response to the treatment;
    - statistically significant changes of glutathione S-transferase (GST) level were not found during the process of cervical cancer treatment.

5. LIST OF SCIENTIFIC PUBLICATIONS AND PRESENTATIONS ON THE TOPIC OF THE DISSERTATION

Scientific publications

1. Daukantienė L, Valuckas KP, Aleknavičius A, Pipirienė-Želvienė T. Chemoradiotherapy in the treatment of locally advanced cervical cancer. Lietuvos chirurgija. 2013; 12(3):119–31.

2. Daukantienė L, Valuckas KP, Žilevičienė A, Didžiapetrienė J, Aleknavičius E. Evaluation of efficacy and safety of neoadjuvant therapy and subsequent chemoradiotherapy for treatment of locally advanced cervical cancer *(in Lithuanian)* Lietuvos akušerija ir ginekologija. 2014; 17(2):106–14.

3. Daukantienė L, Kazbariene B, Valuckas KP, Didžiapetrienė J, Krikstaponiene A, Aleknavičius E. The significance of reduced glutathione and glutathione S-transferase during chemoradiotherapy of locally advanced cervical cancer. Medicina. 2014; 50(4):222–9.

4. Daukantienė L, Schveigert D, Kinderytė E, Fadejeva J, Didžiapetrienė J. Glutathione S-transferase gene polymorphism and cervical cancer *(in Lithuanian)*. Laboratorinė medicina. 2014; 16(2):63–7.

Scientific presentations

1. Daukantienė L, Schveigert D, Kinderytė E, Fadejeva J, Didžiapetrienė J. Study of GSTM1 and GSTT1 polymorphism in cervical cancer patients. 6th Baltic Congress of Oncology. October 3 – 4, 2014. Vilnius, Lithuania.
2. Daukantiene L, Valuckas KP, Kazbariene B, Krikstaponiene A, Didziapetriene J. The impact of antioxidative status for cervical cancer carcinogenesis and its predictive significance during neoadjuvant chemotherapy and chemoradiotherapy of locally advanced cervical cancer. 6th Baltic Congress of Oncology. October 3 – 4, 2014. Vilnius, Lithuania.

6. SUMMARY IN LITHUANIAN

Darbo aktualumas

Gimdos kaklelio vėžys pasaulyje yra ketvirta pagal susirgimų dažnį moterų onkologinė liga ir ketvirta pagal dažnį moterų mirties nuo vėžio priežastis bei pagal sergamumo dažnį antra jaunų moterų vėžio forma. Kasmet pasaulyje diagnozuojama daugiau nei 500 tūkst. naujų gimdos kaklelio vėžio atvejų, iš kurių daugiau nei 85 proc. nustatomi ekonomiškai silpnose šalyse [http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx.]. Nacionalinio vėžio instituto (buvusio Vilniaus universiteto Onkologijos instituto) duomenimis, Lietuvoje 2011 metais gimdos kaklelio vėžys buvo ketvirta pagal sergamumo dažnį bei pagal mirčių nuo vėžio dažnį moterų onkologinė liga, o 30–54 metų amžiaus moterų grupėje gimdos kaklelio vėžys užėmė antrą vietą pagal susirgimų dažnį ir mirtingumą nuo vėžio [http://www.nvi.lt].

Gimdos kaklelio vėžiui susiformuoti turi reikšmės virusiniai, genetiniai, etniniai, aplinkos veiksniai, mitybos įpročiai bei gyvenimo būdas [Wang SS, 2009].

Svarbi vieta formuojantis įvairių lokalizacijų, tarp jų ir gimdos kaklelio vėžiui, tenka oksidacinių-redukcinių procesų pokyčiams organizme, kuriuos parodo lipidų peroksidacijos intensyvumas bei antioksidacinės sistemos fermentų aktyvumas. Padaugėjus laisvųjų radikalų (LR) ir susilpnėjus antioksidacinės sistemos pajėgumui juos neutralizuoti, vystosi oksidacinis stresas. LR perteklius pažeidžia nukleorūgštis, baltymus, slopina fermentus, inicijuoja lipidų peroksidacijos grandinines reakcijas, sutrikdo ląstelės viduląstelinio signalo perdavimo kelius, ir taip daro įtaką kancerogenezei iniciacijos ir promocijos stadijose [Reuter S. et al., 2010].

Onkologiniams pacientams oksidacinio streso pokyčius gali stimuliuoti ir pagilinti, be kitų veiksnių, ir spindulinis gydymas bei chemoterapija, kurie tiesioginiu ar netiesioginiu būdu padidina LR kiekį ląstelėse [Noqueira V. et al., 2013]. Antra vertus, antioksidacinės sistemos būklę atspindintys rodikliai organizmo lygiu yra reikšmingi siekiant prognozuoti ligos eigą bei numatyti atsaką į gydymą [Huang G. et al., 2007].

Vienas pagrindinių antioksidantų ir detoksikuojančių agentų yra redukuotas glutationas (GSH), kuris reguliuoja kitų antioksidantų veiklą, detoksikuoja kenksmingus junginius, moduliuoja imuninės sistemos veiklą ir uždegiminių procesų vystymąsi, dalyvauja transportuojant aminorūgštis per ląstelės membraną, padidina toksiškų medžiagų ar vaistų tirpumą vandenyje sudarydamas su jais konjugatus, yra kai kurių fermentinių reakcijų koveiksnys [Traverso N. et al., 2013]. GSH kaip substratą naudoja fermentai, tarp jų ir glutationo S-transferazė (GST).

Formuojantis navikui svarbus vaidmuo tenka tiek pirminių, tiek ir antrinių antioksidacinių fermentų aktyvumui, tarp kurių yra GST, dalyvaujanti prijungiant toksiškus junginius prie glutationo. Viena vertus, sumažėjus GST fermento aktyvumui, sumažėja organizmo geba metabolizuoti kancerogenus ir tai padidina citogenetinių pažaidų atsiradimo kiekį ir vėžio išsivystymo riziką. Kita vertus, esant dideliam GST fermentų aktyvumui greičiau metabolizuojami vaistai, kas sumažina citotoksinės terapijos poveikį navikinėms ląstelėms ir tai gali būti susiję su blogesniu atsaku į gydymą bei trumpesne pacientų išgyvenimo trukme [Peklak-Scott C. et al., 2005].

Atlikta tyrimų, kurių metu nustatyti GSH ir GST lygiai įvairių lokalizacijų piktybiniais navikais, tarp jų ir gimdos kaklelio vėžiu, sergančiųjų kraujyje bei naviko audiniuose [Beevi SS. et al., 2007; Martens JE. et al., 1997]. Apžvelgtos literatūros duomenys liudija, jog nustatyti reikšmingi šių lygių skirtumai sergančiųjų vėžiu ir sveikų asmenų kraujyje, taip pat nustatyta GSH ir GST lygių kraujyje skirtumų sąsaja su įvairiais klinikiniais, tokiais kaip vėžio stadija, veiksniais bei pokyčiai gydomojo proceso (chemoterapijos, spindulinio gydymo) metu, kas gali būti reikšminga tiek prognoziniu, tiek ir predikciniu aspektais [Sharma A. et al., 2010].

Nustatyta sąsaja tarp GST genų polimorfizmo ir krūties, viršutinių kvėpavimo takų, plaučių, šlapimo pūslės ir priešinės liaukos vėžio išsivystymo rizikos [Di Pietro G. et al., 2010]. Nėra daug duomenų apie GST genų polimorfizmo ir gimdos kaklelio vėžio ryšį. Apie 50 proc. europietiškos kilmės asmenų nustatytas visiškas *GSTM1* aktyvumo praradimas ir apie 25 proc. asmenų – *GSTT1* aktyvumo praradimas (iškrita, *null* genotipas), susijęs su homozigotinio geno delecija [Piacentini S. et al., 2011].

Taigi, atsižvelgiant į GST fermento aktyvumo bei jį koduojančių genų polimorfizmo, taip pat šio fermento substrato GSH svarbą formuojantis įvairių lokalizacijų vėžiui bei galimą jų prognozinę ir predikcinę vertę, yra pagrįsti tolesni jų vaidmens formuojantis gimdos kaklelio vėžiui tyrimai.

Nepaisant atliekamos patikros programos dėl gimdos kaklelio patologijos ir taikomų profilaktinių priemonių, apie 50 proc. atvejų diagnozuojamas jau vietiškai išplitęs IIB–IVA stadijos gimdos kaklelio vėžys [http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx., http://www.nvi.lt.]. Vietiškai išplitusio gimdos kaklelio vėžio gydymo standartas yra suderintas chemospindulinis gydymas cisplatinos pagrindu, tačiau gydymo rezultatai išlieka nepatenkinami, dažnas tiek lokalus, tiek atokus ligos atkrytis. Tai skatina ieškoti naujų, efektyvesnių gydymo galimybių – mėginama įdiegti į praktiką naujus citostatikus, taikinių terapijos preparatus, atliekami klinikiniai tyrimai, kurių metu kartu su spinduliniu gydymu naudojami nauji citostatikų deriniai be cisplatinos ar nauji deriniai cisplatinos pagrindu, kurie taikomi kaip neoadjuvantinė ar adjuvantinė terapija, mėginami nauji citostatikų vartojimo būdai, režimai, dozės. Siekiant individualizuoti gydymą, ieškoma naujų prognozinių ir predikcinių žymenų, kurie įgalintų suskirstyti pacientes į grupes pagal ligos agresyvumą ir leistų parinkti kuo efektyvesnę gydymo taktiką. Neabejotiną prognozinę reikšmę turi jau žinomi klinikiniai ir patologiniai veiksniai: ligos stadija, naviko dydis, sritinių limfmazgių būklė, histologinis tipas, piktybiškumo laipsnis, invazija į limfagysles ir kraujagysles, hipoksija, hemoglobino koncentracija kraujo serume. Tačiau ir esant identiškiems duomenimis tarp pacienčių ligos atsakas į gydymą bei išgyvenimo trukmė dažnai būna nevienoda. Tai skatina tolesnius mokslinius tyrinėjimus, besiremiančius molekulinės biologijos, genetikos pagrindais.

Darbo tikslas

Nustatyti antioksidacinės sistemos veiklą sąlygojančių molekulinių (redukuoto glutationo (GSH), glutationo S-transferazės (GST)) ir genetinių (glutationo S-transferazės (GST) genų polimorfizmo) veiksnių svarbą formuojantis gimdos kaklelio vėžiui bei įvertinti šių veiksnių reikšmę taikant vietiškai išplitusiam gimdos kaklelio vėžiui gydyti neoadjuvantinę chemoterapiją ir chemospindulinį gydymą.

Darbo uždaviniai

1. Nustatyti redukuoto glutationo (GSH) ir glutationo S-transferazės (GST) lygį moterų, sergančių invaziniu vietiškai išplitusiu gimdos kaklelio vėžiu, moterų, kurioms diagnozuoti intraepiteliniai gimdos kaklelio gleivinės pakitimai (CIN3/CIS), ir sveikų moterų kraujo serume.
2. Nustatyti glutationo S-transferazės genų (*GSTM1* ir *GSTT1*) polimorfizmo pasiskirstymo dažnį tarp moterų, sergančių invaziniu vietiškai išplitusiu gimdos kaklelio vėžiu, tarp moterų, kurioms diagnozuoti intraepiteliniai gimdos kaklelio gleivinės pakitimai (CIN3/CIS), ir tarp sveikų moterų.
3. Įvertinti pacienčių, sergančių IIB–IIIB stadijų gimdos kaklelio vėžiu, gydymo efektyvumą ir saugumą taikant intensyvią neoadjuvantinę chemoterapiją cisplatinos ir gemcitabino deriniu bei chemospindulinį gydymą (naudojant tų pačių citostatikų derinį).
4. Nustatyti redukuoto glutationo (GSH) ir glutationo S-transferazės (GST) lygių pokyčius gydomojo proceso metu bei susieti juos su gimdos kaklelio naviko ir organizmo klinikinėmis ir patologinėmis charakteristikomis, atsaku į gydymą bei laikotarpiu iki prasidedant ligos progresavimui.

Ginamieji teiginiai

1. Redukuoto glutationo (GSH) ir glutationo S-transferazės (GST) lygiai yra skirtingi moterų, sergančiųjų invaziniu vietiškai išplitusiu gimdos kaklelio vėžiu, moterų, kurioms nustatyti intraepiteliniai gimdos kaklelio gleivinės pakitimai (CIN3/CIS), bei sveikų moterų kraujo serume. Esant glutationo S-transferazės (GST) genų polimorfizmui, gimdos kaklelio vėžio išsivystymo tikimybė yra didesnė.
2. Intensyvus kassavaitinis neoadjuvantinės chemoterapijos cisplatina ir gemcitabinu kursas prieš chemospindulinį gydymą (naudojant tų pačių citostatikų derinį) yra efektyvus ir saugus gydant vietiškai išplitusiu IIB–IIIB stadijų gimdos kaklelio vėžiu sergančias pacientes.
3. Redukuoto glutationo (GSH) ir glutationo S-transferazės (GST) lygių pokyčių dinamika gydant vietiškai išplitusį gimdos kaklelio vėžį yra reikšminga numatant gydymo efektyvumą.

Darbo mokslinis naujumas

1. Moksliniai - klinikiniai oksidacinio streso tyrimai gimdos kaklelio ikinavikinių ligų bei vėžio atveju nėra gausūs, jų duomenys dar nėra susisteminti. Turimi tyrimų rezultatai apie sąsają tarp glutationo S-transferazės (GST) genų polimorfizmo ir gimdos kaklelio vėžio kontroversiški, nedaug duomenų turima apie *GSTM1*, *GSTT1* genų polimorfizmo įtaka gimdos kaklelio vėžio rizikai Europos populiacijose. Mūsų atlikto palyginamojo tyrimo metu nustatyti redukuoto glutationo (GSH) bei glutationo S-transferazės (GST) lygių kraujo serume statistiškai reikšmingi skirtumai tarp sergančiųjų invaziniu vietiškai išplitusiu gimdos kaklelio vėžiu, tarp moterų, kurioms diagnozuoti intraepiteliniai gimdos kaklelio gleivinės pakitimai (CIN3/CIS), bei sveikų moterų rodo antioksidacinės sistemos komponentų svarbą gimdos kaklelio kancerogenezėje, o statistiškai reikšmingas iškritų *GSTM1* ir *GSTT1* genuose dažnis tarp minėtų grupių leidžia teigti, kad, esant minėtų genų polimorfizmui, tikimybė susirgti gimdos kaklelio vėžiu yra padidėjusi.

2. Vietiškai išplitusiam gimdos kaklelio vėžiui gydyti taikytas naujas intensyvios neoadjuvantinės chemoterapijos cisplatina ir gemcitabinu ir vėliau chemospindulinio gydymo (šiam naudojant tuos pačius citostatikus) derinys. Nedaug žinoma apie oksidacinio streso rodiklių pokyčius gydomojo gimdos kaklelio vėžio proceso metu. Remiantis mūsų atlikto tyrimo duomenimis, galima teigti, jog redukuoto glutationo (GSH) lygio pokyčiai gydomojo gimdos kaklelio vėžio proceso metu gali būti reikšmingi numatant gydymo efektyvumą.

Išvados

1. Nustatyti statistiškai reikšmingi redukuoto glutationo (GSH) ir glutationo S-transferazės (GST) lygių kraujo serume skirtumai tarp moterų, kurioms diagnozuoti intraepiteliniai gimdos kaklelio gleivinės pakitimai (CIN3/CIS), moterų, sergančiųjų invaziniu vietiškai išplitusiu gimdos kaklelio vėžiu, ir sveikų moterų.
2. Glutationo S-transferazės (GST) genų *GSTM1* ir *GSTT1* iškritos sveikoms moterims nebuvo nustatytos. Pacientėms, kurioms nustatyti intraepiteliniai gimdos kaklelio gleivinės pakitimai (CIN3/CIS), iškritų dažnis tirtuose genuose nebuvo ženklus. Didžiausias iškritų dažnis nustatytas pacientėms, sergančioms invaziniu vietiškai išplitusiu gimdos kaklelio vėžiu.
3. Gydant vietiškai išplitusiu IIB–IIIB stadijų gimdos kaklelio vėžiu sergančias pacientes nustatyta:
    - trumpas ir intensyvus kassavaitinis neoadjuvantinės chemoterapijos cisplatina ir gemcitabinu kursas prieš chemospindulinį gydymą yra efektyvus ir saugus;
    - po neoadjuvantinės chemoterapijos atliekamas chemospindulinis gydymas (naudojant tų pačių citostatikų derinį) yra efektyvus, tačiau toksinis poveikis kraujodarai jo metu riboja viso pagal protokolą numatyto gydymo citostatikais realizavimą.

4. Tiriant redukuoto glutationo (GSH) ir glutationo S-transferazės (GST) lygių pokyčius gydomojo gimdos kaklelio proceso metu nustatyta:
    - statistiškai reikšmingi GSH lygio pokyčiai nustatyti priklausomai nuo gimdos kaklelio vėžio stadijos, sritinių limfmazgių būklės bei teigiamo atsako į gydymą;
    - statistiškai reikšmingų GST lygio pokyčių gydomojo gimdos kaklelio vėžio proceso metu nenustatyta.

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