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Clinical Significance of the Notch Signaling Pathway in Uterine Cancer

SUMMARY OF DOCTORAL DISSERTATION

Medical and Health Sciences,
Medicine M 001

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Notch signalinio kelio klinikinė svarba gimdos kūno vėžio atveju

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ABBREVIATIONS

ADAM	–	Disintegrin and metalloproteinase domain-containing protein
AXIN2	–	Axin-like protein 2 gene
BMI	–	Body mass index
cDNA	–	Copy DNA
CTNNB1	–	β-catenin
DLL	–	Notch ligand (Delta-like ligand)
DNA	–	Deoxyribonucleic acid
FIGO	–	International Federation of Gynecology and Obstetrics
FZD	–	Frizzled receptor
G	–	Grade of tumor differentiation
HES1	–	Gene of hairy and enhancer of split-1, Notch pathway component/target, suppressor of transcription preferentially binding to promoter non-canonical N-box
HEY1	–	Gene of hairy and enhancer of split-1, Notch pathway component/target, suppressor of transcription preferentially binding to promoter canonical E-box
Hh	–	Hedgehog signaling pathway
HPRT1	–	Hypoxanthine phosphoribosyltransferase gene
JAG	–	Notch ligand (Jagged)
mRNA	–	Messenger RNA
Notch	–	Notch signaling pathway
PSEN	–	Presenilin
PSENEN	–	Presenilin enhancer 2 homolog
RNA	–	Ribonucleic acid
p	–	Level of significance
PCR	–	Polymerase chain reaction
Wnt	–	Wnt (Wingless and Int-1) signaling pathway

1. INTRODUCTION

Uterine cancer is the sixth most common cancer among women worldwide. In 2018, over 380,000 new cases of uterine cancer were diagnosed [Bray F. et al., 2018]. The highest incidence rate is recorded in North America and Europe, while the lowest – in Africa and Asia. Uterine cancer is the most common malignant tumor of female genital tract in Western countries, with approximately 170,000 new cases diagnosed each year [Torre L.A. et al., 2015]. The incidence of uterine cancer is increasing. This is thought to be related to the current epidemic of obesity, increased life expectancy, reduced fertility, hormone replacement therapy, especially without progestin [Kitchener H.C. et al., 2009; Lortet-Tieulent J. et al. 2018].

Approximately 600–700 cases of uterine cancer are diagnosed in Lithuania every year. It is the third most common malignancy in women after skin (except melanoma) and breast cancer. The incidence of uterine cancer in Lithuania is also increasing [https://www.nvi.lt/uploads/pdf/Vezio%20registras/Vezys_lietuvoje_2012.pdf; https://ecis.jrc.ec.europa.eu].

The relapse and progression of uterine cancer lead to poor prognosis, and the side effects of modern cytostatics often result in discontinuation of treatment. In addition, resistance to conventional treatment is observed in cases of later stages of the disease and in tumor recurrences. There are no approved biological therapies for the treatment of uterine cancer. These factors explain why much focus is recently laid on molecular genetic research in an attempt to identify the genetic disorders affecting the carcinogenesis of the uterine, and which markers can help to predict the course of the disease and to choose individual treatment.

In recent years, studies linking the formation of tumor cells with dysregulation of signaling pathways in that cells leading to malignancy have intensified. Notch signaling is one of such pathways. The signal transmitted through the Notch signaling pathway receptors

in combination with various cellular components influences cell differentiation, proliferation and apoptosis [Morgan T.H., 1917; Lai E.C., 2004; Gordon W.R. et al., 2008; Kopan R. et al., 2009]. Although the function of many newly identified proteins in the Notch signaling pathway is not clear enough, the proteins that form the basis of this signaling pathway are receptors and ligands. Notch receptors are the transmembrane proteins that interacts with adjacent cells by receiving a signal and then transmitting it to the nucleus. Mammals have been found to have four Notch receptors (NOTCH1–4). Notch ligands are also the transmembrane proteins involved in cell interactions that send a signal to the receptor. Mammals have five NOTCH ligands – DLL1, DLL3, DLL4 (delta-like) and JAG1, JAG2 (jagged). Notch targets include the best characterised families of transcriptional repressors *HES* and *HEY* genes. The Notch signaling pathway is proved to be closely related to other oncogenic signaling pathways, including Wnt (a name derived from the names of the *Wingless* and *Int-1*), whose components (CTNNB1, AXIN2, etc.) play an important role in clonogenicity of tumor cells [Bray S.J., 2006; Fiuza U.M. et al., 2007; Gordon W.R. et al., 2008; Kopan R. et al., 2009; Ranganathan P. et al., 2011].

A partial loss of Notch function or abnormal activation of the Notch signal has been shown to be associated with various human developmental disorders and diseases. Mutations in the genes encoding components of the Notch signaling pathway lead to congenital disorders [Meester J.A.N., 2019].

The evidence that the Notch signaling pathway is involved in carcinogenesis is growing. Notch signaling pathway can play a dual role in cancer development: as an oncogene and as a tumor suppressor [Leong K.G. et al., 2006]. The effect of the Notch signal depends on the tumor cell and the type of tissue. Altered expression of certain components of the Notch signaling pathway correlates with poorer prognosis and shorter survival rates in cases of patients with breast, pancreatic, ovarian, prostate, and other cancers [Santagata S. et al.,

2004; Doucas H. et al., 2008; Yuan X. et al., 2015; Chen C. et al., 2017; Pancewicz-Wojtkiewicz J. et al., 2017; Wang J.W. et al., 2018; Wang M. et al., 2018; Zou B. et al., 2018].

Changes in the Notch signaling pathway expression in uterine cancer have received little scholarly attention. Individual literature data indicate that Notch signaling pathway components are involved in the development of uterine cancer [Suzuki T. et al., 2000; Cobellis L. et al., 2008], but research in this area is fragmented as only data concerning isolated components of this signaling pathway have been published. The relationship between the Notch signaling pathway components and the clinical-pathological characteristics of the tumor and the organism has also been scarcely investigated. Further research in this area is needed to reveal the significance of the components of the Notch signaling pathway in the development and progression of uterine cancer, as well as to determine their potential implications for the survival of patients.

1.1 Aim of the study

To determine the expression of the Notch signaling pathway components (receptors, ligands and targets) in tumor and adjacent normal uterine tissue and to evaluate their importance for the survival of uterine cancer patients.

1.2 Objectives of the study

1. To compare the expression of the Notch signaling pathway components at the level of mRNA and protein in tumor and normal uterine tissues.
2. To determine the expression of the Notch signaling pathway components at the level of mRNA and protein at different stages of uterine cancer, degree of tumor differentiation, histologic type, lymphovascular invasion and myometrial invasion.

3. To evaluate the influence of the Notch signaling pathway components on survival of patients with uterine cancer.
4. To carry out multivariate analysis and identify statistically significant prognostic factors.

1.3 Statements to be defended

1. The expression of the Notch signaling pathway components is different in tumor and normal uterine tissues.
2. The expression of the Notch signaling pathway components is related to the clinical-pathological characteristics and survival of patients with uterine cancer.

1.4 Scientific novelty and practical relevance of the study

1. The analysis of the expression of the Notch signaling pathway components was performed by comparing the tumor and normal uterine tissues of the same patient, which is important in order to individually evaluate the changes in the expression of these pathway components and their importance for survival of uterine cancer patients.
2. The study identified a prognostic significance of the Notch signaling pathway components for survival of patients with uterine cancer.
3. Less extensive inhibition of *NOTCH2* gene expression is a negative prognostic factor and can be used in predicting prognosis of the disease in patients with uterine cancer.

2. SUBJECTS AND STUDY METHODS

2.1 Subjects and their characteristics

This prospective observational study enrolled 109 patients who were diagnosed with uterine cancer from 2010 through 2016.

Prior to carrying out the study, the permission was obtained from the Vilnius Regional Biomedical Research Ethics Committee (Protocol No. I-2010-1, issue No. 158200-05-180-43). All samples were collected with the patients' written consent to participate in the study.

The study used operative material obtained at the clinic of Vilnius University Institute of Oncology (now The National Cancer Institute) from patients with stage I−IV uterine cancer. At the time of removal of the uterus, 109 samples of malignancies and 109 samples of normal (surrounding) uterine tissues from the same patients were obtained and histologically examined.

The mean age of the women included in the study was 65.2±9.2 years (range 43−81). The majority of patients were 60−69 years old and 70−79 years old − 33.9% of each group (n = 37); the age group 50−59 − 23.9% (n = 26). The smallest proportion of subjects were women aged 40−49 (3.7%, n = 4) and 80−89 years (4.6%, n = 5).

The majority of patients were postmenopausal patients (92.7%, n = 101), only 7.3% (n = 8) of them were premenopausal subjects.

The mean body mass index (BMI) of the subjects was 33.8±7.2 kg/m^2 (19.4 to 62.4 kg/m^2). Most patients − 72.5% (n = 79) were obese women (BMI 30.0 kg/m^2 and above), 19.3% (n = 21) of subjects were overweight (BMI 25.0−29.9 kg/m^2) and only 8.2% of women (n = 9) were of normal body weight (BMI 18.5−24.9 kg/ m^2). The majority of subjects were diagnosed with primary arterial

hypertension (68.8%, n = 75). Only a small proportion of patients had diabetes (8.3%, n = 9).

All the patients in the study underwent surgical treatment: removal of the uterus (hysterectomy), including adnexa of the uterus, and pelvic and para-aortic lymphadenectomy according to indications. The stage of the disease was determined by the International Federation of Gynecology and Obstetrics (FIGO 2009) and by the Tumor, Nodes, Metastasis (TNM) staging system (2010). The majority of women had endometrioid adenocarcinoma of 82.6% (n = 90), other morphologic variants of uterine cancer were less frequently diagnosed − serous adenocarcinoma (8.3%, n = 9) and carcinosarcoma (9.2%, n = 10). In all, 84 (77.1%) patients were diagnosed with cancer of stage I, 8 (7.3%) − stage II, 11 (10.1%) − stage III and 6 (5.5%) − stage IV. Clinical-pathological characteristics of patients are presented in Table 1.

Table 1. **Clinical-pathological data of patients participating in the study.**

Characteristics	Number of patients (%)
FIGO stage	
IA	51 (46.8)
IB	33 (30.3)
II	8 (7.3)
IIIA	1 (0.9)
IIIB	1 (0.9)
IIIC	9 (8.3)
IVB	6 (5.5)
Histologic type of tumor	
Endometrioid adenocarcinoma	90 (82.6)
Serous adenocarcinoma	9 (8.3)
Carcinosarcoma	10 (9.2)
Tumor differentiation grade	
G1	38 (34.9)
G2	38 (34.9)
G3	28 (25.7)
Undetermined grade	5 (4.6)
Metastasis to regional lymph nodes	
Yes	11 (10.1)
No	98 (89.9)
Lymphovascular invasion	
Yes	18 (16.5)
No	91 (83.5)
Myometrial invasion	
< 1/2	51 (46.8)
≥ 1/2	58 (53.2)
Menopausal status	
Premenopausal	8 (7.3)
Postmenopausal	101 (92.7)
Body mass index	
18.5–24.9	9 (8.3)
25.0–29.9	21 (19.3)
≥30	79 (72.5)

FIGO, International Federation of Gynecology and Obstetrics; IA, tumor limited to the endometrium or invades less than half of the myometrium; IB, tumor invades half or more of the myometrium; II, tumor has spread from the body of the uterus and is growing into the supporting connective tissue of the

cervix, but it has not spread outside the uterus; IIIA, tumor involves the serosa of the corpus uteri and/or adnexa of the uterus; IIIB, The cancer has spread to the vagina or to the tissues around the uterus (the parametrium); IIIC, metastasis to pelvic and/or para-aortic lymph nodes; IVB, distant metastasis; G1, well-differentiated tumor; G2, moderately differentiated tumor; G3, poorly differentiated tumor; < 1/2, tumor invades less than half of the myometrium; ≥ 1/2, tumor invades half or more of the myometrium.

Depending on the stage of the disease and the degree of tumor differentiation, some patients (n = 56) underwent postoperative adjuvant radiotherapy according to the National Cancer Institute standards of treatment: some with vaginal brachytherapy (n = 28), others with combined radiation therapy, i.e. pelvic external-beam radiation therapy along with vaginal brachytherapy (n = 28). A single dose (SD) for pelvic external-beam radiation therapy was administered – 1.8–2.0 Gy in 23–28 fractions five days per week, total dose (TD) 46.0-50.4 Gy. Vaginal (intracavitary) brachytherapy was applied once a week, with 5 Gy (SD) at 0.5 cm depth in the vaginal wall during each brachytherapy procedure, using an iridium-192 source. There were three procedures combined with external-beam radiation therapy (TD – 15 Gy) and four procedures with brachytherapy alone (TD – 20 Gy).

Chemotherapy with cisplatin (50mg/m^2) or carboplatin dose of AUC 5 (area under the curve) in combination with doxorubicin (50mg/m^2) was administered to 16 patients: five post-operative patients received palliative chemotherapy alone for distant metastases, while the remaining patients underwent chemotherapy before or after radiation therapy.

2.2 Study methods

2.2.1 Anthropometric measurements

Body mass index was calculated for all subjects by dividing body mass by height squared (m^2). According to WHO recommendations, patients are normal in body mass with a BMI between 18.5 and 24.9 kg/ m^2, overweight with a BMI of 25.0 to 29.9 kg/ m^2, and obese if the BMI is 30 kg/ m^2 or more [http://www.euro.who.int/en/health-topics/disease-prevention/nutrition/a-healthy-lifestyle/body-mass-index-bmi].

2.2.2. Used equipment

- Homogenizer Mikro-Dismembrator S (Sartorius);
- Centrifuge 5804 (Eppendorf, USA);
- NanoDrop 2000 spectrophotometer (Thermo Scientific, USA);
- Shaker MS 3 basic (IKA®, Germany);
- Thermocycler – Mastercycler (Eppendorf, Germany);
- Thermomixer – Termomixer comfort (Eppendorf, Germany);
- Quantitative PCR system – StepOnePlus™ Thermocycler (Applied Biosystem, USA) or Mastercycler ep realplex (Eppendorf, Germany).

2.2.3 Isolation of RNA

Samples of normal and pathological uterine tissues were frozen in liquid nitrogen and stored at -80 ° C temperature. Part of the tissues frozen in nitrogen was crushed in a Mikro-Dismembrator S (Sartorius) homogenizer according to the manufacturer's recommendations, the rest – manually in a chilled ceramic crucible. Homogenized tissues were lysed with cell lysis buffer. About half of the lysate was left for protein extraction and the remainder for RNA

extraction according to the manufacturer's recommendations (PARIS™ Kit, Ambion). DNase (Thermo Fisher Scientific) was used to remove DNA impurities in purified RNA samples. The resulting RNA solutions were frozen at -80°C. The RNA concentration and purity were determined spectrophotometrically using $OD_{260/280}$ (optical density) and $OD_{260/240}$ ratios. The concentration of patient samples for quantitative PCR testing was standardized prior to carrying out copy DNA synthesis.

2.2.4 Copy DNA (cDNA) synthesis

DNA copy was synthesized using the High Capacity RNA to cDNA Master Mix (Life Technologies) and Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo scientific) according to the manufacturer's recommendations. A similar amount of total RNA from each sample was used to synthesize copy DNA. The synthesized cDNA is stored at -20 ° C temperature until use.

2.2.5 Quantitative PCR

The gene expression research was performed using oligonucleotides Gene expression assays (Table 2) and TaqMan Gene Expression Master Mix (Life Technologies) of PCR. The composition of 20 µl chain reaction mix: 9 µl of five-fold diluted cDNA, 1 µl of oligonucleotides and 10 µl of PCR mix. The reaction temperature mode: initial denaturation at 95 ° C for 10 min.; the following steps repeated 45 times: 95 ° C for 15 sec. and 60 ° C for 1 min. The cDNA level of the tested gene was normalized according to two housekeeping genes *HPRT1* (the hypoxanthine phosphoribosyltransferase 1 gene) and *B2M* (the beta-2-microglobulin gene). On the basis of laboratory experience and the results of the first experimental studies, it was found that the expression of these genes is constant.

The relative change in gene expression (compared to control) was calculated by the Comparative C_t method, i.e. the fold change of gene expression equal to $2^{-\Delta\Delta C}{}_t$, where $\Delta\Delta C_t$ is:

$(C_{t\ test\ gene} - C_{t\ housekeeping\ gene})_{\ tumor\ tissue} - (C_{t\ test\ gene} - C_{t\ housekeeping\ gene})_{\ normal\ tissue}$.

The results are presented as fold change of gene expression normalized to housekeeping genes expression, average of three measurements. The quantitative PCR system was used in the study – thermal cycler StepOnePlus™ (Applied Biosystem, USA) or Mastercycler ep realplex (Eppendorf, Germany).

Table 2. **Oligonucleotide primers used for quantitative PCR.**

Gene	**Number**	**Number of mRNA**
HPRT1	Hs02800695_m1	NM_005524.3
B2M	Hs00984230_m1	NM_004048.2
DLL1	Hs00194509_m1	NM_005618.3
JAG1	Hs 01070032_m1	NM_000214.2
JAG2	Hs 00171432_m1	NM_002226.3
NOTCH1	Hs 01062014_m1	NM_017617.3
NOTCH2	Hs 01050702_m1	NM_001200001.1
NOTCH3	Hs 01128541_m1	NM_000435.2
NOTCH4	Hs 00965889_m1	NM_004557.3
HEY1	Hs0114113_m1	NM_001040708.1
HES1	Hs00172878_m1	NM_005524.3
AXIN2	Hs00610344_m1	NM_004655.3
CTNNB1	Hs00355045_m1	NM_001904.3

2.2.6 Quantitative PCR using arrays

The Human Notch signaling pathway RT² Profiler PCR Array (SABiosciences) was used in the study. In all, 84 tested genes were analyzed. Five housekeeping genes – *B2M, HPRT1, RPL13A* (ribosomal protein L13a gene), *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase gene), *ACTB* (beta cytoskeletal actin gene) were used

for comparison. Positive and negative controls were performed in the analysis. cDNA was synthesized from 1 μg of isolated RNA using RT^2 First strand Kit (SABiosciences) according to the manufacturer's recommendations. Quantitative PCR was performed using the fluorescent dye SYBR Green Mastermix (SABiosciences). The relative change in gene expression was calculated using the manufacturer's recommended analysis software (PCRArrayDataanalysis V3.3 Sofware, SABiosciences) based on five housekeeping genes mentioned above.

2.2.7 Western blot analysis

Protein concentration was determined by bisinchoninic acid (BCA) method. 30 μg of protein was used for the experiment. Proteins were fractionated in 12 percent NDS-polyacrylamide gel under denaturing conditions. Electrophoresis was performed at 80 V, a current of 150 mA for 1 hour, and at 120 V, a current of 150 mA for 3 hours. A semi-dry transfer apparatus V20-SDB was used to transfer the proteins onto the nitrocellulose membrane (0.45 μm). The transfer was carried out for 1 h at a current of 1 mA/cm2. The membrane was then incubated with rabbit monoclonal antibodies anti-NOTCH1 (1:200 dilution; sc-9170, Santa Cruz Biotechnology, or 4147S, Cell Signaling), anti-NOTCH4 (1:200 dilution; sc-5594, Santa Cruz Biotechnology), anti-HES1 (PA5-28802, Thermo Scientific), anti-JAG2 (1: 300; # 2210, Cell Signaling), with anti-non-phospho β-catenin antibodies (8814S, Cell Signaling), and mouse monoclonal anti-β-actin antibodies (1:1000; sc-8432, Santa Cruz Biotechnology, or MA5-15739, Thermo Scientific) overnight at +4°C temperature. These antibodies were the only choice at the start of the study, while others such as anti-NOTCH2 were not available at the time.

The membranes were further incubated for 2 hours with alkaline phosphatase-conjugated secondary antibodies against rabbit or mouse IgG (1:1000 dilution; # 18-732-292604, GenWay Biotech or sc-2008, Santa Cruz Biotechnology, respectively). Proteins were

expressed using a mixture of NBT (nitromel tetrazole) and BCIP-T (5-brome-4-chlore-3-indolyl phosphate, p-toluidine salt). Membrane-bound primary antibodies NICD1 (NOTCH intracellular domain1), HES1 (hairy and enhancer of split-1) and β-catenin were detected using horseradish peroxidase-conjugated anti-rabbit IgG secondary antibodies (31460, Thermo Scientific). β-actin antibodies were detected using horseradish peroxidase-conjugated anti-mouse IgG secondary antibodies (31430, Thermo Scientific). The NOTCH protein content was normalized to β-actin content. The relative amount of NOTCH protein is the ratio of the amount of protein in tumor tissue to the amount of protein in normal tissue.

2.2.8 Evaluation of protein expression

The ImageJ program evaluated the protein strip area and color intensity. The relative amount of test protein was normalized to the relative amount of β-actin ($\frac{the\ intensity\ of\ the\ specific\ protein\ band\ of\ the\ sample}{the\ intensity\ of\ the\ \beta-\text{actin band of the same sample}}$).

The change in protein expression is expressed multiplied by times, comparing the expression of the test protein in tumor and normal tissues.

2.2.9 Statistical analysis of the data

Calculation of the sample. It is estimated that 84 clinical samples are required to detect a significant correlation (if any) with a maximum low correlation coefficient (0.3) and choosing a criterion power of 0.8 and a significance level of 0.05. A power criterion of 0.8 and a significance level of 0.05 were also used to calculate the sample needed for the analysis of variance; it is estimated that if the difference between the mean values of the samples is equal to or greater than 1, with a standard deviation of 0.5, at least 7 samples from each group will be required.

To evaluate the clinically meaningful effect on patient survival, a total of 92 samples (46 in each group) were prognozed, where 30% difference between survival rates was considered clinically significant along with power criterion 0.8 and significance level 0.05.

The sample size was also calculated taking into account that about 140 new cases of uterine cancer are diagnosed at the National Cancer Institute (former Vilnius University Institute of Oncology) per year. Planning that about 65 percent of patients will be excluded from the study for a variety of reasons (e.g., failure to meet inclusion criteria, insufficient volume of tumor and normal uterine tissue, surgical treatment) and taking into account that up to 10% extreme values can be included in the study, it was foreseen that the study shall include about 25 percent of the patients who visited the National Cancer Institute during the study period, i.e. about 35 patients a year.

The sample size was calculated so that, at a significance level of 0.05, the power of the criterion was sufficient to detect clinically significant changes. When the *P* value was between 0.05 and 0.1, the differences were considered to be tendentious. Data normality was assessed using the Shapiro–Wilk W test. The Mann–Whitney U test was used to compare two independent groups, and Kruskal–Wallis dispersion analysis was used to compare several independent groups. The correlation analysis was performed using Spearmen correlation coefficient. All experimental data were used for statistical analysis, but extremes are not presented in graphical representations. The extremes accounted for less than 10% of the total data. One-factor survival analysis was performed using the Log-rank test. COX regression analysis was used in conducting the multivariate survival analysis.

When the *P* value was 0.05 to 0.1, the differences between the groups were considered to be trendy, and when the *P* value was less than 0.05, the differences were considered statistically significant.

Statistically significant results were visualised using box-plots and survival curves in the summary of doctoral dissertation.

SigmaPlot 13.0 and Statistica Basic Academic 13 were used for data analysis.

Most of the experiments were performed at the Life Sciences Center of Vilnius University under the supervision of dr. Violeta Jonušienė (quantitative PCR), dr. Aušra Sasnauskienė (Western blot immunoassay), dr. Daiva Dabkevičienė (statistical data analysis). The author is grateful for their valuable contribution.

3. RESULTS

3.1 Study plan

Tumor and normal uterine tissue samples from 109 patients obtained after surgery were used for research. The expression of Notch signaling pathway receptors (*NOTCH1-4*), ligands (*JAG1, JAG2, DLL1*) and targets (*HES1, HEY1*) genes was assessed by quantitative PCR.

In the analysed samples, the expression of NOTCH1 (n = 70), NOTCH4 (n = 75), and HES1 (n = 41) was assessed at protein level. In the samples of some patients (n = 59), the gene expression of the regulatory factor *AXIN2* (n = 59) and the transduction factor *CTNNB1* (n = 59), as well as CTNNB1 (n = 32) protein level of the components of the WNT pathway closely related to the Notch signaling pathway, were also evaluated. The study of the Wnt signaling pathway components was purely exploratory and of limited scope, therefore it is excluded from the aim of the dissertation, its objectives, titles of the sections and conclusions, however, we hope that the obtained results will be useful for further work in this direction.

The expression of the above Notch and Wnt signaling pathway components was not investigated in all samples due to the insufficient amount of available tumor and normal (control) tissue required to obtain reliable results.

Subsequently, 10 patient samples of tumor and normal tissues were selected because of they were of sufficient size to allow more extensive analysis. In these samples, 84 genes were analyzed by quantitative PCR.

The results of mRNA assays are presented *as fold changes in gene expression*, normalized to housekeeping gene expression (detailed in the Study methods section). It is important to note that for the descriptive statistics, the mean values of the study data series – the medians – were used.

3.2 Changes in the expression of components of the Notch signaling pathway depending on the stage of the disease

The study found that the expression of *NOTCH1-4* genes in the tumor tissue was decreased compared to normal uterine tissue in the same patient, median values < 0 in all cases. The Kruskal–Wallis test was used to compare the expression of Notch signaling pathway receptors genes between stages I and II–IV, regardless of the histologic type of tumors, i.e. all malignancy histologic types were included. There is no statistically significant difference in the expression of *NOTCH1–4* genes, in all cases $p > 0.05$.

The study found that the expression in Notch signaling pathway ligands *JAG1, JAG2, DLL1*, targets *HES1, HEY1* genes and the expression of regulatory factor *AXIN2* and transduction factor *CTNNB1* of the WNT signaling pathway in tumor tissues compared normal tissues from the same patient is also reduced in most cases, with median values < 0 in all cases. The Mann–Whitney test was used for comparison of the expression of ligands *JAG1, JAG2, DLL1*, targets *HES1, HEY1* genes, and regulatory factor *AXIN2* and transduction factor *CTNNB1* of the WNT signaling pathway, between stages I and II–IV, irrespective of tumor histology, i.e. all tumor histologic types were included. The results showed that there was no statistically significant difference in the expression of the mentioned genes, in all cases $p > 0.05$.

Changes in the expression of receptors *NOTCH1−4*, ligands *JAG1, JAG2, DLL1*, targets *HES1, HEY1* genes, *AXIN2, CTNNB1* components of the Wnt pathway were evaluated according to the histologic type of malignancies separately in each case of endometrioid adenocarcinoma, carcinosarcoma, serous adenocarcinoma. The data analysis did not show statistically significant differences in the gene expression between the various stages of these histologic types of tumors. In the case of carcinosarcoma and serous adenocarcinoma, the absence of

statistically significant differences may be explained by a small number of subjects.

Although no significant changes in the expression of *NOTCH1-4*, *JAG1*, *JAG2*, *DLL1*, *HES1*, *HEY1*, *AXIN2*, *CTNNB1* genes were observed depending on the stage of the disease, however, the majority of the samples corresponded to stages IA and IB (77.1%), therefore an assumption was made that the gene expression may differ between stages IA and IB and additionally only stages IA and IB were compared.

Comparing the mRNA expression between stages IA and IB in all histologic types, there was a statistically significant difference in *NOTCH4* gene expression between samples at stages IA and IB (Figure 1A). There was also a significant difference in *HEY1* gene expression (Figure 1B). The results show that the expression of *NOTCH4* and *HEY1* genes at the level of stage IB is lower than at stage IA, i.e. in a more advanced disease, the expression of *NOTCH4* and *HEY1* genes at the transcript level is suppressed.

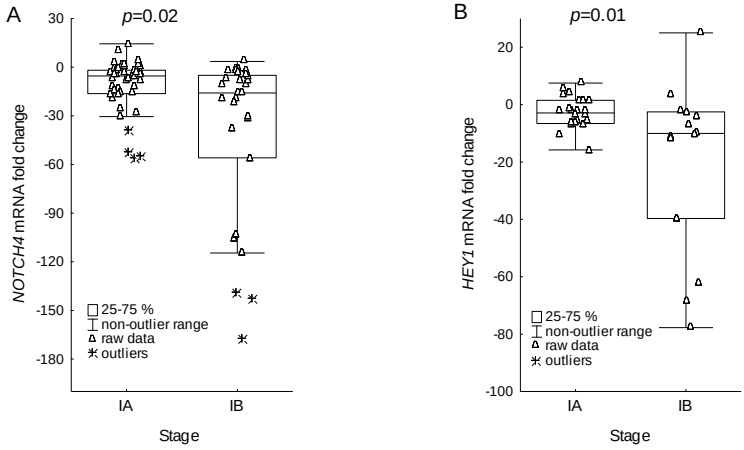

Figure 1. Changes in *NOTCH4* (A) and *HEY1* (B) genes expression in tumor tissue compared to normal tissue regardless of the histologic type of tumor at stage I

The study also evaluated fold changes of the expression of *NOTCH1-4*, *JAG1*, *JAG2*, *DLL1*, *HES1*, *HEY1*, *AXIN2*, *CTNNB1* genes between stages IA and IB for each of the three histologic types separately.

The largest group of patients (n = 90) consisted of women diagnosed with endometrioid adenocarcinoma, including 70 cases with stage I. These patients were divided into two groups: stage IA (41 patients) and stage IB (29 patients). The statistical analysis revealed that the *NOTCH4* gene expression was significantly lower in the histologic tissue of the examined tumor in stage IB patients than in the corresponding tumor tissues of stage IA (Figure 2A). In endometrioid adenocarcinoma, statistically significant differences in *HEY1* gene expression between stage IA and IB were also found (Figure 2B). In stage IB, the gene expression in tumor tissues was more decreased when compared to normal tissues in stage IA.

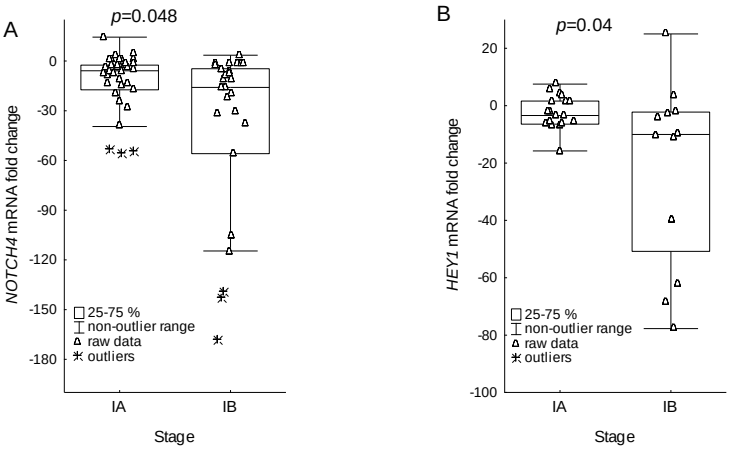

Figure 2. Changes in *NOTCH4* (A) and *HEY1* (B) genes expression in tumor tissue compared to normal tissue in stage I endometrioid adenocarcinoma.

In serous adenocarcinoma, there was a trend for *NOTCH1* gene expression to be lower in stage IB than in stage IA (p=0.08).

After the changes in the gene expression of the components of Notch and Wnt signaling pathways had been examined, further research focused on changes at the protein level.

After carrying out the Western blot analysis, changes in NOTCH1 and NOTCH4 expression at the protein level were assessed in all the samples of tumor and normal uterine tissues. HES1 and transduction factor CTNNB1 protein levels were detected in the later enrolled patients (n = 59). Protein levels were normalized to β-actin. A significant change is determined where more than a double increase or decrease in the protein level is observed.

The data of our study revealed that NOTCH1, NOTCH4, HES1 and CTNNB1 protein levels in tumor tissues varied within a relatively small interquartile range: medians (interquartile range) were 1.15 (2.04), 1.03 (2.96), 0.4 (2.25) and 1.03 (2.96), respectively, compared to normal tissues. However, the full range of meanings was quite wide: 17, 11, 33, 55, respectively. It is important to note that NOTCH1 protein level tended to increase with the increasing stage of the disease, while NOTCH4 tended to decrease with the increasing stage of the disease, particularly in IA transition to IB. The Mann–Whitney test was used to compare the level of NOTCH1 protein between stages I and II–IV, irrespective of the histologic type of tumor (Figure 3). The results revealed that the level of NOTCH1 protein is significantly higher in stages II–IV compared to protein levels observed in I stage samples (p=0.02).

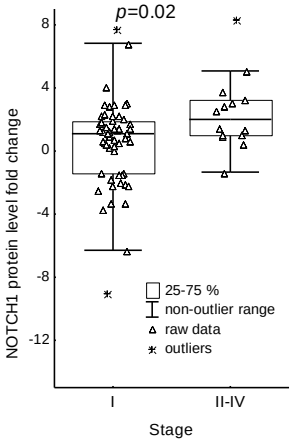

Figure 3. Change in NOTCH1 protein level depending on the disease stage.

As with the identification of gene expression, the differences in the protein levels of the receptors NOTCH1 and NOTCH4, the target HES1 and the transduction factor CTNNB1 as the component of the Wnt signaling pathway were evaluated separately in samples from patients with stage IA and IB uterine cancer. NOTCH4 protein level was found to be significantly lower in stage IB compared to protein level found in IA stage samples (Figure 4).

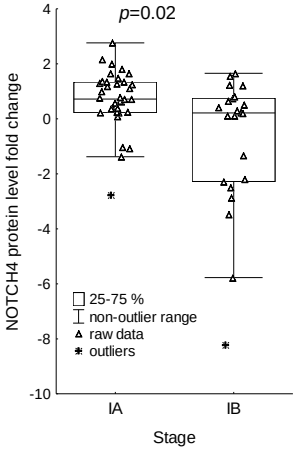

Figure 4. Change in NOTCH4 protein level regardless of the histologic type of tumor at stage I.

A separate analysis of different histologic types of tumors showed a statistically significant decrease in the level of receptor NOTCH4 protein at stage IB compared to that of stage IA (p=0.01).

The evaluation of the levels of other proteins (HES1 and CTNNB1) did not detect a statistically significant difference between the various stages of uterine cancer.

We further analyzed the expression changes of the components of Notch and Wnt signaling pathways at mRNA and protein levels with respect to other clinical-pathological characteristics – grade of tumor differentiation, histologic type, lymphovascular and myometrial invasion.

3.3 Changes in the expression of components of the Notch signaling pathway depending on grade of tumor differentiation

The results of this study fragment showed that, depending on the degree of differentiation, a statistically significant difference in the target *HEY1* and Wnt pathway component *AXIN2* gene expression was found – the expression of poorly differentiated tumors is significantly

lower compared to well- and moderately differentiated tumors (Figure 5).

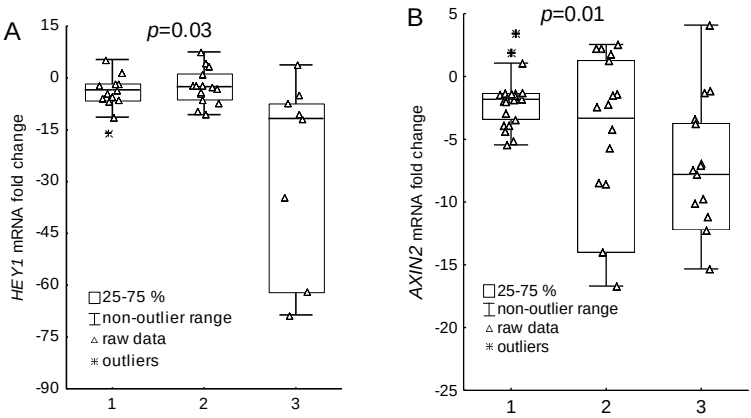

Figure 5. Changes of *HEY1* (A) and *AXIN2* (B) genes expression in tumor tissue compared to normal tissue depending on the degree of tumor differentiation (1 – well-differentiated tumor; 2 – moderately differentiated tumor; 3 – poorly differentiated tumor).

There is a trend for *NOTCH4* gene expression to be lower in moderately and poorly differentiated tumors compared to well-differentiated ones (Figure 6A). Similar changes observed when analyzing NOTCH4 protein expression are given in Figure 6B.

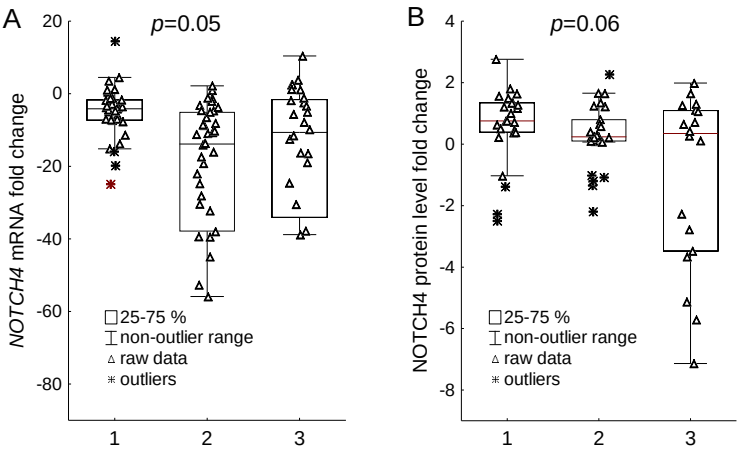

Figure 6. Changes of *NOTCH4* (A) gene expression and NOTCH4 protein level (B) in the tumor tissue compared to normal tissue depending on the degree of tumor differentiation (1 – well-differentiated tumor; 2 – moderately differentiated tumor; 3 – poorly differentiated tumor).

The analysis showed that there is a trend of *DLL1* gene expression to decrease in poorly differentiated uterine tumors compared to well-differentiated ones (p=0.09).

3.4 Changes in the expression of components of the Notch signaling pathway depending on tumor histologic type

The research into this fragment disclosed a trend for *NOTCH2* gene expression to be lower in endometrioid adenocarcinoma than in carcinosarcoma or serous adenocarcinoma samples (p=0.09). The analysis of the Wnt signaling pathway component *AXIN2* gene expression the showed that the expression is the lowest in serous adenocarcinoma samples, but in this case the sample size is too small to state significant changes.

Thus, it is clear from the results presented that no statistically significant differences in the expression of gene and the levels of

protein were observed between the histologic types of tumors. This may have been influenced by insufficient carcinosarcoma and serous adenocarcinoma samples.

3.5 Changes in the expression of components of the Notch signaling pathway depending on lymphovascular and myometrial invasion

The research into this fragment did not reveal any statistically significant differences depending on whether lymphovascular invasion was present in the tumor or not, but a trend was observed that NOTCH1 protein level (p=0.1), *JAG1* (p=0.08) and *DLL1* (p=0.10) genes expression in the presence of tumor lymphovascular invasion is higher compared to samples where lymphovascular invasion was not determined.

Changes in *NOTCH4* gene expression and NOTCH4 protein level were statistically significant, depending on tumor invasion of the myometrium – if the tumor infiltrates half or more than half myometrium, the expression of *NOTCH4* gene and NOTCH4 protein level in uterine tissue is lower than when the tumor infiltrates less than half myometrium. The results obtained are shown in Figure 7.

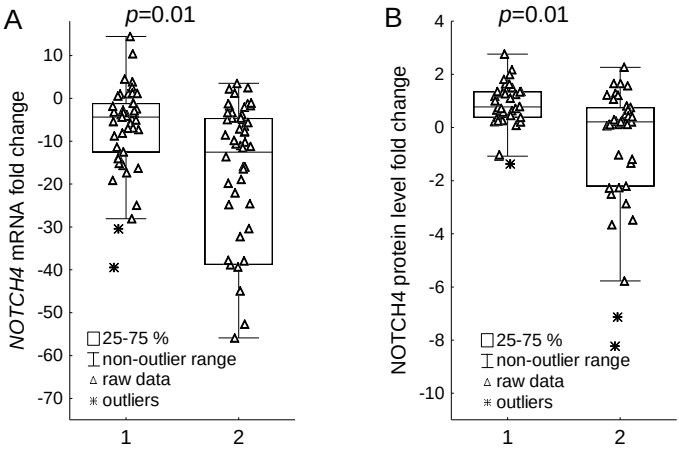

Figure 7. Changes of *NOTCH4* gene expression (A) and NOTCH4 protein level (B) depending on the myometrial invasion (1 – < 1/2 myometrium; 2 – = or > 1/2 myometrium).

3.6 Changes in the expression of the Notch signaling pathway components depending on patient age and the evaluation of correlation between the investigated parameters

On the one hand, no statistically significant differences were found between the results obtained in different age groups, and on the other hand, a trend was observed for *JAG2* ligand gene expression to be lower in tumors of after-surgery patients over 65 years of age compared to younger patients (Figure 8).

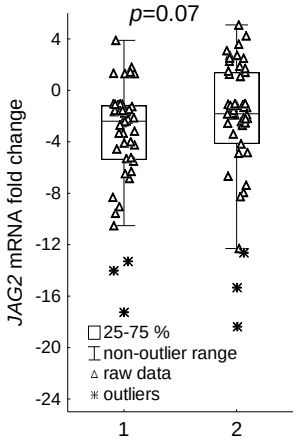

Figure 8. Change of *JAG2* gene expression in tumor tissue compared to normal tissue with respect to patient age (1 – to 65 years, inclusive; 2 – over 65 years).

The study also evaluated the correlation between gene expression of the Notch and Wnt signaling pathways and protein levels in the samples tested. Using the Spearmen correlation coefficient, a positive correlation was found between many genes and proteins. Correlation coefficients were significant at $p < 0.05$ as shown in Table 3. A statistically significant correlation was found between different Notch signaling pathway receptors, between receptors and ligands, and between components of the Notch and Wnt signaling pathways. After evaluating the correlation between clinical-pathological data and the test molecules, a positive correlation was found between *AXIN2* gene expression and body weight (correlation coefficient 0.31, $p<0.05$).

Table 3. **Evaluation of the correlation between the components of the Notch and Wnt signalling pathways. Only statistically significant ($p < 0.05$) correlation coefficients differing from 0 are presented in the table. Proteins (NOTCH4, HES1, CTNNB1) that did not correlate with all investigated components are not included in the table.**

	NOTCH1 **mRNA**	**NOTCH1 protein**	***NOTCH2*** **mRNA**	***NOTCH3*** **mRNA**	***NOTCH4*** **mRNA**	***JAG1*** **mRNA**	***JAG2*** **mRNA**	***DLL1*** ***m*****RNA**	***HES1*** **mRNA**	***HEY1*** **mRNA**	***AXIN2*** **mRNA**	***CTNNB1*** **mRNA**
NOTCH1 **mRNA**			0.75	0.75	0.75	0.75	0.79	0.77	0.77	0.55	0.48	0.40
NOTCH1 protein							-0.28		-0.30			
NOTCH2 **mRNA**	0.75			0.76	0.74	0.71	0.64	0.76	0.73	0.51	0.42	0.61
NOTCH3 **mRNA**	0.75		0.76		0.66	0.72	0.66	0.68	0.71	0.50	0.43	0.54
NOTCH4 **mRNA**	0.75		0.74	0.66		0.67	0.65	0.76	0.63	0.48	0.45	0.37
JAG1 **mRNA**	0.75		0.71	0.72	0.67		0.74	0.67	0.73	0.65		0.38
JAG2 **mRNA**	0.79	-0.28	0.64	0.66	0.65	0.74		0.70	0.67	0.65		
DLL1 **mRNA**	0.77		0.76	0.68	0.76	0.67	0.70		0.71	0.58	0.57	0.34
HES1 **mRNA**	0.77	-0.30	0.73	0.71	0.63	0.73	0.67	0.71		0.64	0.32	0.33
HEY1 **mRNA**	0.55		0.51	0.50	0.48	0.65	0.65	0.58	0.64			
AXIN2 **mRNA**	0.48		0.42	0.43	0.45			0.57	0.32			0.30
CTNNB1 **mRNA**	0.40		0,61	0.54	0.37	0.38		0.34	0.33		0.30	

3.7 Changes in gene expression of the Notch signaling pathway components in tumor tissue as determined by PCR arrays

The samples of the ten patients. with sufficient amount of tumor and normal tissues were analysed using Human Notch signaling pathway RT² Profiler PCR Array. The changes in Notch signaling pathway and related gene expression were assessed using the Human Notch signaling pathway RT² Profiler PCR Array. Five samples of endometrioid adenocarcinomas and five samples of carcinosarcoma and their corresponding normal tissue samples were examined. The changes in the expression of some genes involved in maturation of Notch receptors, as well as WNT and Hedgehog signaling pathways in stage I malignant uterine cells are shown in Figure 9. The results show that expression of genes involved in Notch receptor's maturation in tumor tissues is reduced: *ADAM10* (ADAM metalopeptidase 10 domain), *PSEN1* (presenilin 1), *PSEN2* (presenilin 2), *PSENEN* (presenilin enhancer) (Figure 9A). The study also found that the expression of components of the Hedgehog (Hh) signaling pathway is reduced or remains unchanged: expression of SHH ligand, transcription factor GLI1 (GLI family zinc finger 1), SUFU (suppressor of fused homolog) was reduced in tumor tissue, compared to normal tissue, whereas the expression of SMO (smoothened, frizzled family receptor) and GSK3B (glycogen synthase kinase 3 beta) remained unchanged (Figure 9B). Reduced levels of the mRNA of the following proteins were observed in tumor uterine tissue samples: Wnt signaling pathway receptor *FZD1* (frizzled family receptor 1), *FZD2, FZD3, FZD4, FZD6, FZD7* and other components' AES (amino-terminal enhancer of split), *AXIN1*, *CTNNB1* (catenin beta 1), *LRP5* (low density lipoprotein receptor-related protein 5), *WISP1* (WNT1 inducible signaling pathway), *WNT11* (Wingless-type MMTV integration site family, member 11). The expression of the *TLE1* (transducin-like enhancer of split 1) remained unchanged (Figure 9C).

The observed differences in gene expression of components of these signaling pathways in tumor tissues and normal tissues indicate the relevance of further studies into these signaling pathways in order to identify new molecular markers of uterine cancer.

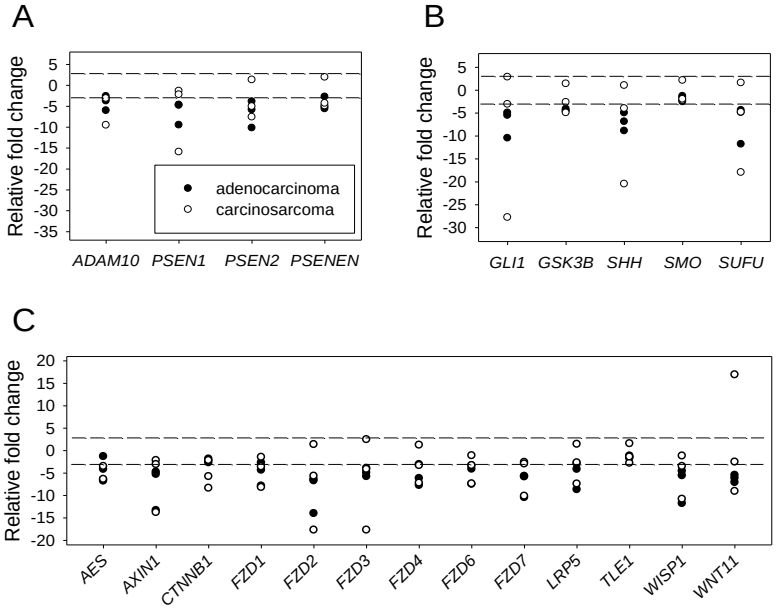

Figure 9. Amounts of transcripts of different signaling pathways (Notch signaling pathway (A), Hh pathway components (B) and Wnt signaling pathway (C)) components. Hollow characters – carcinosarcoma, filled characters – adenocarcinoma. The expression was considered to be significantly altered when it was reduced or increased more than three-fold (dashed line).

3.8 Patient survival depending on clinical-pathological characteristics and expression of the Notch signaling pathway components

Patients were enrolled in the study between 2010 and 2016 and monitored. Censorship date is 1 March 2019.

The analysis of the data showed that in the studied group of women with uterine cancer, females who, when diagnosed, were 65 years of age or younger had a significantly longer survival rate than older patients (Figure 10).

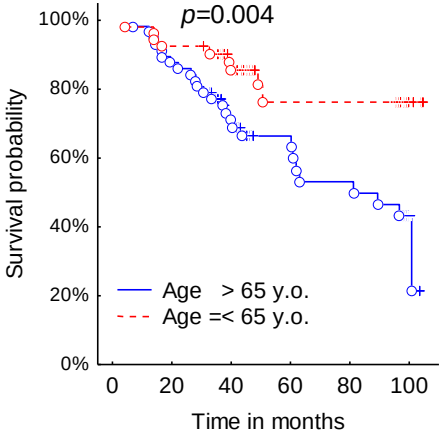

Figure 10. Age-related survival of patients with uterine cancer; o – fatal cases, + censored cases.

As expected, the disease stage significantly influenced the survival rate – with locally advanced or metastatic tumor of the uterus (stage II–IV of the disease), the survival was worse than with stage IA–IB, where the tumor did not spread beyond the uterine body (Figure 11).

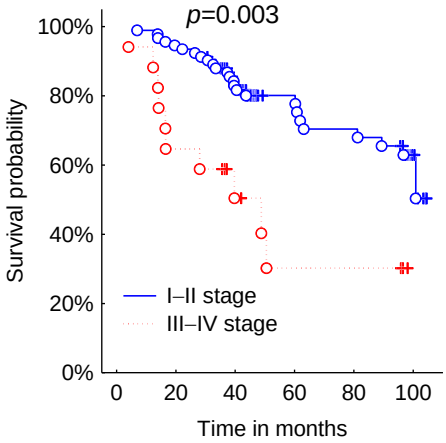

Figure 11. Survival rates in patients with uterine cancer depending on the stage of the disease; o – fatal cases, + censored cases.

Patients with histologically confirmed endometrioid adenocarcinoma had a better survival rate than those with other histologic forms – carcinosarcoma and serous adenocarcinoma (Figure 12).

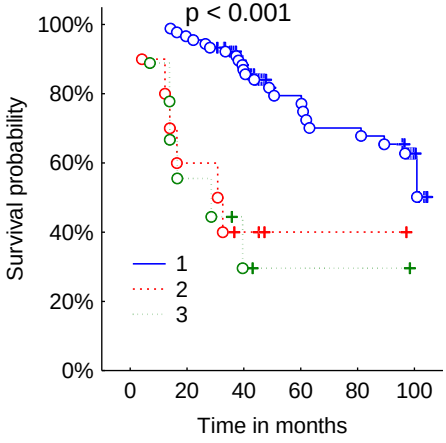

Figure 12. Survival rate in patients with uterine cancer depending on the histologic type of tumor: 1 – endometrioid adenocarcinoma; 2 – carcinosarcoma; 3 – serous adenocarcinoma; o – fatal cases, + censored cases.

The degree of tumor differentiation was a significant determinant for survival - with the lowest degree of tumor differentiation (G3, poorly differentiated tumor) the worst survival results were obtained (Figure 13).

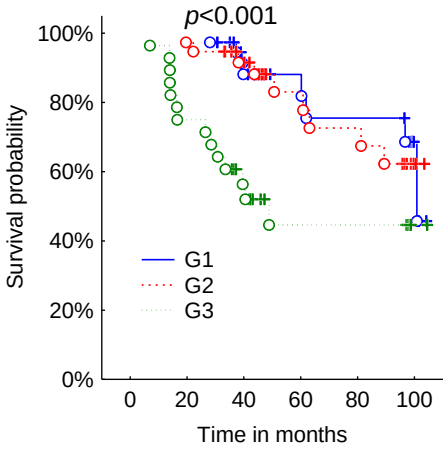

Figure 13. Survival rates of uterine cancer patients depending on the degree of tumor differentiation (G1, well-differentiated tumor; G2, moderately differentiated tumor; G3, poorly differentiated tumor); o – fatal cases, + censored cases.

The survival rates also statistically significantly depended on tumor lymphovascular invasion – in the absence of lymphovascular invasion the survival results were better compared to the patients with lymphovascular invasion (Figure 14).

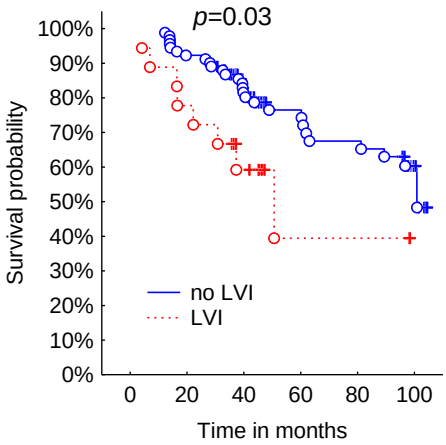

Figure 14. Survival rates in patients with uterine cancer depending on lymphovascular invasion (LVI); o – fatal cases, + censored cases.

In our group of patients with uterine cancer, myometrial invasion had no significant effect on patient survival rates (p=0.83).

Patients who underwent surgery alone had significantly better survival rates than those receiving post-operative radiation therapy or/and chemotherapy (Figure 15).

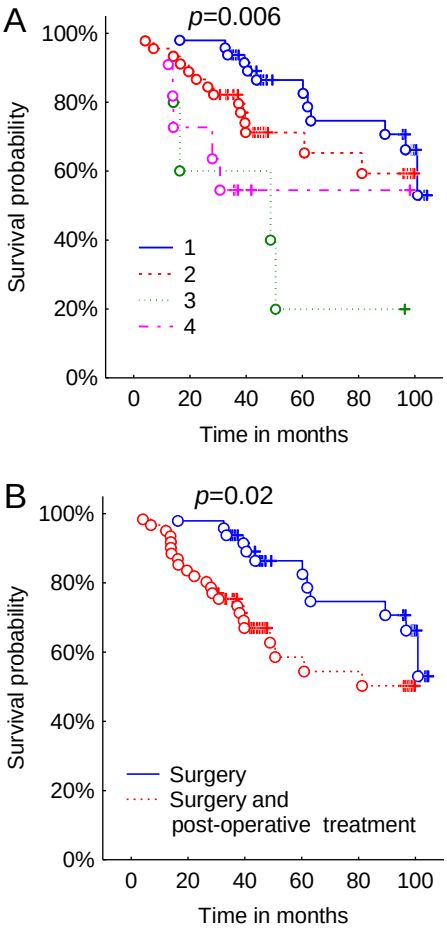

Figure 15. Survival rates of patients with uterine cancer depending on the treatment used (1 – surgery; 2 – surgery followed by radiation therapy; 3 – surgery followed by chemotherapy; 4 – surgery followed by radiation therapy and chemotherapy); o – fatal cases, + censored cases.

The data showed that BMI had no statistically significant effect on patient survival rates (p=0.36).

Thus, the analysis of the data obtained showed that, as expected, the survival rate of patients with uterine cancer is influenced by clinical-pathological characteristics such as stage of disease, histologic type of tumor, degree of tumor differentiation, lymphovascular invasion. The study found that patient survival was undoubtedly dependent on the treatment they underwent, with the best prognosis being in the group of patients who underwent only surgery.

The study evaluated the potential influence of the investigated Notch signaling pathway components on survival rates in patients with uterine cancer. The *P* values are shown in Table 4.

Table 4. **Influence of the Notch signaling pathway components on survival rates in patients with uterine cancer.**

Notch signaling pathway component tested	***P* value**
NOTCH1 receptor gene	0.59
NOTCH1 receptor protein	0.23
NOTCH2 receptor gene	***0.002***
NOTCH3 receptor gene	0.33
NOTCH4 receptor gene	0.64
NOTCH4 receptor protein	0.998
JAG1 ligand gene	0.66
JAG2 ligand gene	0.98
DLL1 ligand gene	0.078
HES1 target gene	0.556
HES1 target protein	0.295
HEY1 target gene	0.31

The analysis of the obtained data showed that there is a statistically significant association between the survival rates of uterine cancer patients and the expression of NOTCH2 gene (Figure 16). In case of higher the *NOTCH2* gene expression, the disease

prognosis is worse. There was also a trend for *DLL1* ligand to influence the survival rates (p=0.08).

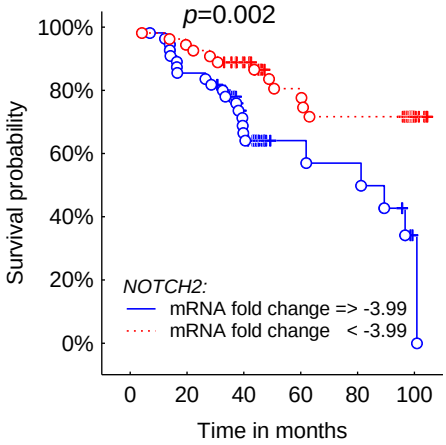

Figure 16. Survival rate of uterine cancer patients depending on the fold change of the *NOTCH2* gene expression; o – fatal cases, + censored cases.

The possible influence of Wnt pathway components closely related to the Notch signaling pathway on the survival rate of patients with uterine cancer has also been identified (Table 5). There is a trend observed from these results that *AXIN2* may influence the survival (p=0.08). Apparently, in order to get reliable results, a larger sample and/or a longer patient monitoring period is needed.

Table 5. **Influence of Wnt signaling pathway components on survival rates of patients with uterine cancer.**

Wnt signaling pathway component tested	***P* value**
AXIN2 gene	0.08208
CTNNB1 gene	0.81667
CTNNB1 protein	0.57615

Multivariate Cox regression analysis was performed to evaluate the significance of clinical-pathological characteristics and their interaction on patient survival rates. Factors that were significant in the one-way analysis were included in the model: disease stage, histologic type, degree of differentiation, lymphovascular invasion, treatment methods, as well as changes in *NOTCH2* receptor and *DLL1* ligand genes expression.

Multivariate Cox regression analysis revealed that histologic type, stage of the disease and changes of *NOTCH2* gene expression were among major determinants of patient survival. The coefficients (Hazard Ratio) of other factors selected in the one-factor survival analysis did not show a statistically significant difference from 1 in the Cox model (Table 6).

Table 6. **Results of multivariate analysis of patients with uterine cancer (only statistically significant results are presented).**

Characteristics	***P* value**	**Hazard ratio**	**95,0% CI* for Hazard ratio**
Fold change of *NOTCH2* expression:			
≥ -3.99	0.004	3.17	1.46–6.89
< -3.99 (reference group)	–	1	–
Histologic tumor type:			
Serous adenocarcinoma/ carcinosarcoma	<0.001	4.35	2.03–9.31
Endometrioid adenocarcinoma (reference group)	–	1	–
FIGO stage:			
III–IV	<0.001	4.31	1.98–9.38
I–II (reference group)	–	1	–

*CI is the confidence interval

The data presented indicate that *NOTCH2* gene expression, along with disease stage and tumor histologic type, are important prognostic factors in uterine cancer.

4. DISCUSSION OF THE RESULTS

Endometrial cells undergo intense expression of the Notch signaling pathway receptors and ligands. This pathway is thought to be important for the endometrial change processes [Mitsuhashi Y. et al., 2012]. NOTCH1 receptor expression in the proliferating endometrium is known to be higher than in other phases, and in the menopausal endometrium, where glandular volume is reduced, mucosa is thinned, protein levels of the Notch signaling pathway are decreased. These findings have led to the theory that the Notch signaling pathway is important for endometrial cell proliferation and the formation of uterine cancer [Cobellis L. et al., 2008].

It was determined that the NOTCH4 receptor is involved in endometrial turnover processes and is important for carcinogenesis [Suzuki T. et al., 2000]. It was also proved that NOTCH1 and JAG1 protein levels are higher in the secretion stage than in proliferation, while NOTCH4 shows reversed results. With reference to literature, an increase in NOTCH1 protein level and a decrease in NOTCH4 and JAG1 protein levels have been observed in tumor cells [Cobellis L. et al., 2008]. However, other authors suggest that the levels of NOTCH1, NOTCH3, DLL4, and JAG1 proteins are the same in both secretory and proliferating endometrium, and therefore they are not important for cell proliferation and differentiation. Elevated levels of these proteins have been detected in tumor cells [Groeneweg J.W. et al., 2014]. It should be noted that all of the above results were obtained by immunostaining of tissues, also by comparing them to the tissues of healthy subjects and cancer patients, and a group of healthy subjects also included younger women.

Our study compared the expression of Notch signaling pathway components in tumor and normal tissues of the same patient's uterine. To our knowledge, this was the first study of the kind at the beginning of our work. It is noteworthy that almost all women in the study were in postmenopause, so menopausal status was not evaluated

by statistical analysis. The majority of the samples (82.6%) consisted of samples of endometrioid adenocarcinoma type I. A relatively small number of samples of other histologic types – of type II cancer (serous adenocarcinoma, carcinosarcoma) and especially a low number of samples of later stages (II–IV) made it difficult to obtain statistically reliable results for these uterine tissues.

Based on our findings, the expression of the Notch signaling pathway components (*NOTCH1−4, JAG1, JAG2, DLL1, HES1, HEY1*) in tumor was decreased compared to their expression in normal uterine tissue of the same patient.

Comparing tumor samples from different histologic types, we found a statistically significant difference in expression between *NOTCH4* receptor and *HEY1* target genes between stages IA and IB, i.e. their expression in stage IB uterine cancer was significantly lower than in stage IA tissues. This was confirmed by analyzing samples of endometrioid adenocarcinomas separately. The data obtained show that the expression of *NOTCH4* and *HEY1* at stage IB is lower than at stage IA, i.e. in a more advanced disease, the expression of *NOTCH4* and *HEY1* genes is suppressed. A significantly reduced *NOTCH4* expression at IB stage of uterine cancer suggests that this gene may be mutated in uterine tumor cells. Mutations in the genes *NOTCH1, NOTCH2, NOTCH3* are found in the head and neck carcinoma cells [Agrawal N. et al., 2011; Stransky N. et al., 2011].

The evaluation of the differences in the expression of the Notch signaling pathway components depending on the clinical-pathological factor such as the degree of tumor differentiation revealed that poorly differentiated tumors have lower levels of *HEY1* gene expression compared to well- and moderately differentiated tumors. A similar trend is observed for *NOTCH4* and *DLL1* expression. The obtained results support the notion that the NOTCH signaling pathway is important for cell differentiation and is involved in carcinogenesis – the reduction of expression of the mentioned Notch pathway components is favorable for carcinogenesis.

The study found a trend for *NOTCH2* receptor gene expression to be lower in endometrioid adenocarcinoma than in samples of carcinosarcoma or serous adenocarcinoma. Taking into account that the prognosis is better in endometrioid adenocarcinoma than in other histologic types, it is possible that the *NOTCH2* gene is less suppressed in tumors with a worse prognosis. In order to obtain statistically significant data, the sample size for carcinosarcomas and serous adenocarcinomas has to be increased.

Another prognostic factor, lymphovascular invasion, is likely to be related to components of the Notch signaling pathway, such as ligands *JAG1* and *DLL1*, but further research is needed in this area.

A significant factor correlating with invasion of the uterine tumor into myometrium was found to be *NOTCH4* gene expression and NOTCH4 protein level. The results obtained from the study confirm the already described scholarly data that the expression of *NOTCH4* is different between tumor samples corresponding to stage IA and IB. Thus, the results of the study confirm the findings of other authors that the Notch signaling pathway may play an important role in the development of uterine cancer and reveal that the expression of relevant Notch signaling pathway genes and protein level may be influential contributors to the development of a particular disease state.

The study has also evaluated the changes in the expression of some components of the Wnt signaling pathway closely related to the Notch signaling pathway, namely, regulatory factor *AXIN2* and transduction factor *CTNNB1*, and their impact on uterine cancer. The analysis of the data obtained revealed that the expression of *AXIN2* and *CTNNB1* in uterine tumor tissue is decreased as compared to normal Notch signaling components in the same patient. In our study we found a statistically significant difference in *AXIN2* gene expression depending on the degree of tumor differentiation, the expression in poorly differentiated tumors is lower compared to well and moderately differentiated tumors. A positive correlation between

expression of *AXIN2* and body weight was found after evaluating the correlation between clinical-pathological data and the molecules tested. These results may lead to the development of further studies on the components of the Wnt signaling pathway.

The present study used PCR arrays to reveal that the amount of mRNA encoding proteins (*ADAM10, PSEN1, PSEN2, PSENEN*) related to Notch receptor maturation in tumor tissue was reduced. The mRNA levels in the Notch signaling pathway targets *HES1* and *HEY1* also decreased in tumor tissue compared to normal tissue. Members of the *HES1* and *HEY1* gene families are transcriptional inhibitors and are primary targets of the Notch signaling pathway, and their reduction in expression reveals that the Notch signaling pathway in tumor tissue is inhibited from the very first components of the signaling pathway.

The expression of some genes in other Wnt and Hedgehog signaling pathways related to the Notch signaling pathway was also investigated. Wnt and Hedgehog signaling pathways are important in embryogenesis and changes in them occur at the presence of various oncological diseases. An abnormal activation of the Hedgehog signaling pathway is important for the proliferation of endometrial carcinoma cells [Hecht J.L. et al., 2006]. The results of our study using PCR arrays also confirm the associations between Notch and other signaling pathways, and the observed differences in gene expression of these signaling pathway components in tumor and normal tissues suggest the relevance of further studies into these pathways in order to identify new molecular markers of uterine cancer.

One of the aims of this study was to evaluate the influence of the Notch signaling pathway components on the survival of uterine cancer patients. Literature data on this topic are very limited. One study [Groeneweg J.W. et al., 2014] found that the prognosis of the disease was influenced by changes in NOTCH1 and JAG1 proteins levels, while another noted that the Notch signaling pathway ligand DLL3 protein may be an independent marker predicting poorer survival rate in patients with uterine cancer [Wang J. et al., 2018]. Our

study did not examine the DLL3 ligand, so this type of study could be one aspect of our future work.

This study aimed at the evaluation of the impact of the Notch and WNT signaling pathway components on patient survival. The influence of clinical-pathological factors on disease prognosis was evaluated as well. As expected, the analysis of the data obtained showed that the survival rate of patients with uterine cancer is affected by the stage of the disease, histologic type of tumor, degree of tumor differentiation, and lymphovascular invasion. In our study, myometrial invasion was not a statistically significant factor influencing patient survival. Patient survival was also found to be dependent on the treatment applied – the best prognosis was found in the group of patients who had received surgery only. This is explained by the fact that additional postoperative treatment (radiation therapy, chemotherapy) is administered in the presence of unfavorable prognosis factors.

The examination of the influence of Notch and Wnt signaling pathway components on patient survival revealed a statistically significant association between the uterine cancer survival rate and *NOTCH2* gene expression: if the *NOTCH2* gene expression is higher, the prognosis of the disease is worse. The weakness of our dissertation is that this study did not investigate NOTCH2 protein level, which should be one of the priority objectives for further research. We also had few cases of serous adenocarcinoma and carcinosarcoma, which did not allow statistically significant evaluation of the *NOTCH2* expression dependence on histologic type. As mentioned above, the results of our study suggest the hypothesis that the Notch signaling pathway is increasingly suppressed in the progression of uterine cancer. Although more detailed studies are needed to explain this phenomenon, the findings of our study indicate the potential prognostic importance of *NOTCH2* expression.

The data of our study suggest that Notch signaling pathway ligand *DLL1* and Wnt pathway component *AXIN2* may also be

important to the survival of patients with uterine cancer, however, due to an insufficient sample of subjects, it was impossible to prove their statistical significance. Therefore, future in-depth research could prove their impact on patient survival.

There is limited evidence in the literature that NOTCH2 receptor is an important prognostic factor. In a recent publication, it has been reported that high levels of *NOTCH2* expression show a statistically significant correlation with poorer survival in ovarian cancer patients [Chen C. et al., 2017]. In 2018, a study was published in which NOTCH2 protein is one of the factors contributing to the prognosis of endometrial cancer [Polychronidou G. et al., 2018]. Thus, our findings that *NOTCH2* gene expression is an important factor in predicting prognosis of the disease in patients with uterine cancer, are relatively recent, and confirm the scientific and practical significance of the studies performed.

5. CONCLUSIONS

1. The Notch signaling pathway is suppressed: expression of components of this pathway in tumor uterine tissue is lower than in normal tissue of the same patient.
2. The expression of Notch signaling pathway components is related to the clinical-pathological characteristics of patients with uterine cancer:
   - for stage IB, the expression of the *NOTCH4* at mRNA and protein level and the expression of the *HEY1* gene are significantly lower than their expression in stage IA;
   - the expression of *HEY1* gene is significantly lower in poorly differentiated tumors as compared to its expression in well- and moderately differentiated tumors;
   - if the tumor has infiltrated half or more than half of the myometrium, the expression of the *NOTCH4* at mRNA and protein level is significantly lower than when the tumor has infiltrated less than half myometrium.
3. A statistically significant association between patient survival and the degree of suppression of *NOTCH2* gene expression was found in uterine cancer survival outcomes: the prognosis is worse with less suppressed *NOTCH2* gene expression in tumor tissue as compared to its expression in normal tissue.
4. Statistically significant independent prognostic factors are *NOTCH2* gene expression, histologic type of uterine tumor, and stage of disease.

6. FUTURE STUDIES

1. In future studies, it is necessary to increase sample size of patients with II–IV stages of uterine cancer and to expand the range of histologic types of tumors.
2. Taking into account the predictive significance of *NOTCH2* gene expression in the survival rate of uterine cancer in the present study, it is appropriate to further investigate changes in the protein content of this gene and to evaluate its significance as a circulating marker.
3. The trend (apparently due to insufficient number of analyzed cases) of the changes of the Notch signaling pathway ligand *DLL1* and the Wnt pathway, closely related Notch signaling pathway (both pathways important for cell differentiation and proliferation), component *AXIN2* genes expression, was identified to influence the survival rate of uterine cancer which is an encouragement to pursue research in this direction.

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

Scientific publications

1. Jonušienė V, Bielskienė K, Lachej N, Kazbarienė B, Mickė I, Didžiapetrienė J. Notch signalinis kelias ir jo įtaka ląstelės likimui. Laboratorinė medicina. 2011; 13(4):199-204.
2. Lachej N, Didžiapetrienė J, Kazbarienė B, Kanopienė D, Jonušienė V. Association between Notch signaling pathway and cancer. Acta Medica Lituanica. 2012; 19(4):427–37.
3. Jonusiene V, Sasnauskiene A, Lachej N, Kanopiene D, Dabkeviciene D, Sasnauskiene S, Kazbariene B, Didziapetriene J. Down-regulated expression of Notch signaling molecules in human endometrial cancer. Med Oncol. 2013; 30(1):438.
4. Lachej N, Dabkevičienė D, Sasnauskienė A, Trimonytė RM, Kanopienė D, Kazbarienė B, Didžiapetrienė J. NOTCH signalinio kelio ir ginekologinių piktybinių navikų sąsaja. Acta Medica Lituanica. 2017; 24(1):35–43.
5. Lachej N, Jonušienė V, Gasianec A, Sasnauskienė A, Dabkevičienė D, Šimienė J, Sužiedėlis K, Didžiapetrienė J. Changes of Notch and Wnt signaling molecules expression in human endometrial cancer. Acta Medica Lituanica. 2019; 3 (*submitted*).

Scientific presentations

1. Lachej N, Jonusiene V, Sasnauskiene A, Dabkeviciene D, Kanopiene D, Didziapetriene J. Down-regulated expression of Notch signalling molecules has prognostic value in human endometrial cancer. The 1st International Doctoral Students' Conference „Science for Health". Kaunas (Lithuania), April 13, 2018.

2. Lachej N, Jonusiene V, Sasnauskiene A, Dabkeviciene D, Gasianec A, Didziapetriene J. The activity of Notch signalling pathway in human endometrial cancer. 14th Baltic Congress of Laboratory Medicine. Vilnius (Lithuania), May 10-12, 2018.

8. SUMMARY IN LITHUANIAN

Pasaulyje gimdos kūno vėžys tarp piktybinių moters navikų užima šeštąją vietą. 2018 metais iš viso diagnozuota daugiau nei 380 tūkst. naujų gimdos kūno vėžio atvejų [Bray F. ir kt., 2018]. Didžiausias sergamumas yra Šiaurės Amerikoje ir Europoje, mažiausias – Afrikos ir Azijos šalyse. Gimdos kūno vėžys yra dažniausias moterų lyties organų piktybinis navikas Vakarų šalyse, kur kasmet diagnozuojama apie 170 tūkst. naujų atvejų [Torre L. A. ir kt., 2015]. Sergamumas gimdos kūno vėžiu didėja. Manoma, kad tai susiję su dabartine nutukimo epidemija, ilgėjančia gyvenimo trukme, vaisingumo sumažėjimu ir hormonų pakaitine terapija, ypač preparatų be progestinų [Kitchener H. C. ir kt., 2009; Lortet-Tieulent J. ir kt., 2018].

Lietuvoje per metus diagnozuojama apie 600–700 gimdos kūno vėžio atvejų. Tai trečias pagal dažnį moterų piktybinis navikas po odos (išskyrus melanomą) ir krūties vėžio. Sergamumas gimdos kūno vėžiu Lietuvoje taip pat didėja [https://www.nvi.lt/uploads/pdf/Vezio%20registras/Vezys_lietuvoje_2012.pdf; https://ecis.jrc.ec.europa.eu].

Gimdos kūno vėžio atkryčiai bei metastazavimas sąlygoja blogą prognozę, o nepageidaujamas šiuolaikinių citostatinių preparatų poveikis organizmui dažnai tampa gydymo nutraukimo priežastimi. Be to, esant vėlesnei ligos stadijai ir naviko recidyvams, stebimas atsparumas tradiciniam gydymui. Registruotų biologinės terapijos preparatų gimdos kūno vėžiui gydyti nėra. Minėti veiksniai paaiškina, kodėl pastaraisiais metais skiriama daug dėmesio molekuliniams genetiniams tyrimams siekiant išsiaiškinti, kokie genetiniai sutrikimai turi įtakos gimdos kūno kancerogenezei, kokie žymenys gali padėti prognozuoti ligos eigą bei parinkti individualų gydymą.

Pastaraisiais metais daugėja tyrimų, siejančių piktybinių ląstelių atsiradimą su signalinių kelių reguliacijos sutrikimais tose ląstelėse. Vienas iš tokių kelių yra Notch signalinis kelias. Signalas,

perduodamas per Notch signalinio kelio receptorius ir veikdamas kartu su įvairiais ląstelės komponentais, daro įtaką ląstelės diferenciacijos, proliferacijos ir apoptozės procesams [Morgan T. H., 1917; Lai E. C., 2004; Gordon W. R. ir kt., 2008; Kopan R. ir kt., 2009]. Nors daugelio naujai identifikuotų baltymų funkcijos Notch signaliniame kelyje nėra visiškai aiškios, galima išskirti baltymus, kurie sudaro šio signalinio kelio pagrindą – tai receptoriai ir ligandai. Notch receptorius yra transmembraninis baltymas, kuris dalyvauja greta esančių ląstelių tarpusavio sąveikoje priimdamas signalą ir toliau perduodamas jį į branduolį. Nustatyta, kad žinduoliai turi keturis Notch receptorius (NOTCH1–4). Notch ligandas taip pat yra transmembraninis baltymas, dalyvaujantis ląstelių sąveikoje ir siunčiantis signalą receptoriui. Žinduoliai turi penkis Notch ligandus – DLL1, DLL3, DLL4 (angl. *delta-like ligand*) ir JAG1, JAG2 (angl. *jagged*). Tarp Notch taikinių yra geriausiai apibūdintos transkripcijos slopiklių *HES* ir *HEY* genų šeimos. Nustatyta, jog Notch signalinis kelias glaudžiai susijęs su kitais onkogeniniais signaliniais keliais, tarp jų ir su Wnt (pavadinimas kilęs iš *Wingless* (liet. besparnis fenotipas) baltymo/integrazės-1), kurio komponentai (CTNNB1, AXIN2 ir kt.) yra svarbūs naviko ląstelių kamieniškumui [Bray S.J., 2006; Fiuza U.M. ir kt., 2007; Gordon W.R. ir kt., 2008; Kopan R. ir kt., 2009; Ranganathan P. ir kt., 2011].

Nustatyta, kad dalinis Notch funkcijos praradimas arba nenormalus Notch signalo aktyvinimas yra susijęs su įvairiais žmogaus vystymosi sutrikimais ir ligomis. Genų, koduojančių Notch signalinio kelio komponentus, mutacijos sukelia įgimtus sutrikimus [Meester J.A.N. ir kt., 2019].

Daugėja įrodymų, kad Notch signalinis kelias dalyvauja kancerogenezėje. Notch signalas, vystantis navikui, gali veikti dvejopai: kaip onkogenas ir kaip naviko augimo slopiklis [Leong K.G. ir kt., 2006]. Notch signalo poveikis priklauso nuo naviko ląstelės ir audinio tipo. Pakitusi tam tikrų Notch signalinio kelio komponentų raiška koreliuoja su blogesne ligos prognoze ir trumpesniu

išgyvenamumu krūties, kasos, kiaušidžių, priešinės liaukos ir kitų lokalizacijų navikų atveju [Santagata S. ir kt., 2004; Doucas H. ir kt., 2008; Yuan X. ir kt., 2015; Chen C. ir kt., 2017; Pancewicz-Wojtkiewicz J. ir kt., 2017; Wang J.W. ir kt., 2018; Wang M. ir kt., 2018; Zou B. ir kt., 2018].

Gimdos kūno vėžio atveju Notch signalinio kelio raiškos pokyčiai yra mažai tirti. Pavieniai literatūros duomenys rodo, kad Notch signalinio kelio komponentai dalyvauja formuojantis gimdos kūno vėžiui [Suzuki T. ir kt., 2000; Cobellis L. ir kt., 2008], tačiau tyrimai šioje srityje yra fragmentiški – paskelbti duomenys tik apie pavienius šio signalinio kelio komponentus. Sąsaja tarp Notch signalinio kelio komponentų ir naviko bei organizmo klinikinių-patologinių charakteristikų taip pat mažai tirta. Reikalingi tolesni šios srities moksliniai tyrimai siekiant patikslinti Notch signalinio kelio komponentų reikšmę formuojantis bei progresuojant gimdos kūno vėžiui, taip pat nustatyti jų galimą reikšmę sergančiųjų išgyvenamumui.

8.1 Darbo tikslas

Nustatyti Notch signalinio kelio komponentų (receptorių, ligandų ir taikinių) raišką navikiniame ir sveikame gimdos kūno audinyje bei įvertinti jų svarbą sergančiųjų gimdos kūno vėžiu išgyvenamumui.

8.2 Darbo uždaviniai

1. Palyginti Notch signalinio kelio komponentų raišką iRNR ir baltymo lygiais navikiniame ir sveikame gimdos kūno audinyje.
2. Įvertinti Notch signalinio kelio komponentų raiškos iRNR ir baltymo lygiais skirtumus esant skirtingoms gimdos kūno vėžio stadijoms, naviko diferenciacijos laipsniui,

histologiniam tipui, limfovaskulinei invazijai bei invazijai į miometriumą.
3. Įvertinti Notch signalinio kelio komponentų raiškos pokyčių įtaką sergančiųjų gimdos kūno vėžiu išgyvenamumui.
4. Atlikti daugiaveiksnę analizę ir nustatyti statistiškai reikšmingus prognozinius veiksnius.

8.3 Ginamieji teiginiai

1. Notch signalinio kelio komponentų raiška skirtinga navikiniame ir sveikame gimdos kūno audinyje.
2. Notch signalinio kelio komponentų raiška susijusi su pacienčių, sergančių gimdos kūno vėžiu, klinikinėmis-patologinėmis charakteristikomis ir išgyvenamumu.

8.4 Darbo mokslinis naujumas ir praktinė reikšmė

1. Notch signalinio kelio komponentų raiška analizuota lyginant tos pačios pacientės navikinį ir sveiką gimdos kūno audinį, kas svarbu siekiant individualizuotai vertinti tirtųjų šio kelio komponentų raiškos pokyčius ir jų svarbą sergančiųjų gimdos kūno vėžiu išgyvenamumui.
2. Nustatyta Notch signalinio kelio komponentų prognozinė reikšmė sergančiųjų gimdos kūno vėžiu išgyvenamumui.
3. Mažiau slopinama *NOTCH2* geno raiška yra neigiamas prognozinis veiksnys ir gali būti pritaikytas kaip prognozinis žymuo parenkant pacientei gydymo ir stebėjimo taktiką.

8.5 Išvados

1. Notch signalinis kelias yra slopinamas: šio kelio komponentų raiška gimdos kūno navikiniame audinyje yra mažesnė, palyginti su jų raiška sveikame tos pačios pacientės audinyje.
2. Notch signalinio kelio komponentų raiška yra susijusi su pacienčių, sergančių gimdos kūno vėžiu, klinikinėmis-patologinėmis charakteristikomis:
    - IB stadijos atveju *NOTCH4* raiška iRNR ir baltymo lygiais bei *HEY1* geno raiška yra statistiškai reikšmingai mažesnė, palyginti su jų raiška IA stadijos atveju;
    - *HEY1* geno raiška blogos diferenciacijos navikuose yra statistiškai reikšmingai mažesnė, palyginti su jo raiška vidutiniškai ir gerai diferencijuotuose navikuose;
    - jei navikas yra infiltravęs pusę ar daugiau kaip pusę miometriumo, *NOTCH4* raiška iRNR ir baltymo lygiais yra statistiškai reikšmingai mažesnė, nei tuo atveju, kai navikas infiltravęs mažiau negu pusę miometriumo.
2. Įvertinus sergančiųjų gimdos kūno vėžiu išgyvenamumo rezultatus priklausomai nuo Notch signalinio kelio komponentų raiškos, nustatyta statistiškai reikšminga sąsaja tarp pacienčių išgyvenamumo ir *NOTCH2* geno raiškos slopinimo laipsnio: esant mažiau slopinamai *NOTCH2* geno raiškai navikiniame audinyje, palyginti su sveiku audiniu, prognozė yra blogesnė.
3. Statistiškai reikšmingi nepriklausomi prognoziniai veiksniai yra *NOTCH2* geno raiška, gimdos naviko histologinis tipas ir ligos stadija.

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