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NOTCH1, NOTCH3, NOTCH4, and JAG2 protein levels in human endometrial cancer

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ABSTRACT

Background and objective: Notch signaling is a conserved developmental pathway, which plays an important role in the regulation of cell proliferation, differentiation and death. Deregulation of Notch pathway has been connected with the carcinogenesis in a variety of cancers. The aim of this study was to investigate the level of the Notch signaling pathway proteins (NOTCH1, 3, 4 and JAG2) in the samples from human endometrial cancer.
Materials and methods: The amount of the Notch receptors NOTCH1, 3, 4 and ligand JAG2 protein was determined by *Western blot analysis* in the samples from stage I endometrial cancer and adjacent nontumor endometrial tissue of 22 patients.
Results: The level of NOTCH4 receptor was 1.7 times lower in stage I endometrial cancer as compared with the healthy tissue of the same patients ($P = 0.04$). The protein level of ligand JAG2 was significantly reduced by 2.5 times in stage IB endometrial adenocarcinoma samples ($P = 0.01$). It was reduced in the majority of stage IB adenocarcinomas. There were no significant changes in the protein amount of NOTCH1 and NOTCH3 receptors comparing stage I endometrial adenocarcinoma and healthy tissues.
Conclusions: The reduced amount of NOTCH4 and JAG2 proteins and the decreased level of mRNA coding Notch proteins, as reported in our previous studies, supports the notion that Notch pathway has rather tumor-suppressive than oncogenic role in human endometrial cancer cells. It suggests that Notch pathway activation is a potential therapeutic target.

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1. Introduction

Endometrial cancer is the most common gynecological malignancy in the western world. Approximately 550 cases per year of endometrial cancer are diagnosed in Lithuania. Cases of endometrial cancer are increasing; it has been related with changes in reproductive behavior, increased obesity, increased life duration and the use of hormone replacement therapy [1]. The majority (70–80%) of endometrial cancer is estrogen-dependent endometrioid type adenocarcinoma (type I endometrial cancer); and the increased incidence of endometrial cancer is confined to this type of cancer. This type of cancer often arises from endometrial hyperplasia of peri- and postmenopausal women. Endometrioid carcinomas are characterized by a variety of genetic alterations, which are affecting distinct genes and signaling pathways [1,2].

The Notch is an evolutionally conserved signaling pathway that has been implicated in a variety of processes, including determination of cell fate, regulation of cell proliferation, differentiation and cell death. The core elements of the Notch signaling pathway in mammals consist of four Notch transmembrane receptors (NOTCH1-4) and 5 transmembrane ligands: three Delta-like proteins (DLL1, 3, 4) and 2 Jagged proteins (JAG1, 2). The receptor and ligand are typically presented on neighboring cells; therefore cell–cell contact is necessary to trigger the signaling event. Ligand binding to its cognate receptor initiates proteolytic cleavage of the receptor by TACE metalloproteinase and γ-secretase which causes the release of intracellular Notch receptor domain. When the intracellular domain translocates into the nucleus, it induces transcriptional activation of Notch target genes [3,4].

Abnormal Notch signaling has been showed in many cancers. Notch pathway can act as an oncogene or as a tumor suppressor and thus can either promote or inhibit tumor cell grow. The outcome of Notch signaling activity depends on signal strength, timing, cell type and context [3]. Upregulated expression of Notch signaling genes was found in many solid tumors, including breast cancer, colorectal cancer, non-small cell lung carcinoma, melanoma and hematological malignancies (reviewed in Ref. [4]). Components of the same pathway may have growth suppressive functions in hematopoietic cells, skin, pancreatic epithelium, and hepatocytes [5].

Notch signaling has been extensively studied in a variety of gynecologic cancers, including ovarian cancer [6] and cervical cancer [7]. There are only few reports concerning Notch signaling in endometrial cancer [8–10]. The amount of Notch signaling molecules determines the strength of the signal since Notch activity does not rely on secondary messengers for signaling amplification [4,11]. Our previous study demonstrated a significant decrease in mRNA level of Notch receptors (NOTCH1, NOTCH2, NOTCH3 and NOTCH4), ligands (JAG1, JAG2 and DLL1) and target gene HES1 [12]. As well, we found significant correlations between transcript amounts of Notch target gene *HES1* and the transcripts of several Notch signaling molecules: NOTCH1, NOTCH2, NOTCH3, JAG2 and DLL1. In this study we examined the protein levels of the Notch receptors NOTCH1, NOTCH3, NOTCH4 and ligand JAG2 in endometrial cancer and adjacent non-tumor endometrial tissue of 22 patients.

2. Materials and methods

2.1. Patients and specimens

Human endometrial samples were obtained from 22 women undergoing surgery during 2010–2011. The age of the patients ranged from 50 to 81 years (mean, 67.8). The clinicopathological characteristics of patients are shown in Table 1. The tissue samples were obtained from the Institute of Oncology, Vilnius University. Specimens were classified by histopathologists. All of these samples had a paired control sample, i.e., adjacent nontumor endometrial tissue. The tissue samples were snap frozen immediately in liquid nitrogen and stored at −80 °C until analysis. Endometrial cancer was staged by the International Federation of Gynecology and Obstetrics (FIGO) staging system [13]. All samples were collected with patient's written informed consent in accordance with ethics approval by the Lithuanian Bioethics Committee.

2.2. Western blot

Frozen tumor and paired normal tissues were homogenized and extracted using The Ambion® PARIS™ system (Life Technologies). Protein concentration in lysate was determined using bicinchoninic acid (BCA) [14]. Samples, containing 30 μg of total protein, were subjected to 12% SDS-PAGE at 120 V. Proteins were transferred to a nitrocellulose membrane (BioRad) by semi-dry blotting. After blocking with 5% BSA, the membrane was incubated with rabbit monoclonal anti-NOTCH1 (1:200; sc-9170, Santa Cruz Biotechnology) or anti-NOTCH3 (1:700; #5276, Cell Signaling), anti-NOTCH4 (1:200; sc-5594, Santa Cruz Biotechnology), anti-JAG2 (1:300; #2210, Cell Signaling) and mouse monoclonal anti-β-actin antibody (1:1000; sc-8432, Santa Cruz Biotechnology) overnight at 4 °C. Membrane attached primary antibodies were detected by incubation for 2 h at 4 °C with alkaline phosphatase conjugated secondary anti-rabbit or

Table 1 – Clinicopathological characteristics of the patients.

Characteristic	Number of patients
FIGO stage	
IA	12
IB	10
Histological type	
Endometrioid adenocarcinoma	22
Grade	
G1	10
G2	10
G3	2
Lymph node metastasis	
(−)	22
Menopausal status	
Premenopausal	1
Postmenopausal	21
Body mass index	
<25 kg/m^2	1
≥25 kg/m^2	21

anti-mouse antibodies (1:1000; #18-732-292604, GenWay Biotech or sc-2008, Santa Cruz Biotechnology, respectively). The immunoreactive bands were visualized exposing the membrane to solution of nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate, p-toluidine salt (BCIP-T). The intensity of the *bands* on a *Western blot was evaluated using ImageJ software. The* amount of Notch signaling protein was normalized to the amount of β-actin. The relative expression of Notch signaling protein was calculated as the ratio of protein amount in the adenocarcinoma versus healthy tissues. The change in the relative amount of protein was considered to be substantial when it was equal or higher than 1.5; when the relative amount was increased or decreased for less than 1.5 times, it was considered as unchanged.

2.3. *Statistical analysis*

Software Sigma Plot 12 was used for statistical analysis. The significance of the difference between protein amount in cancer and normal tissue was determined by the Wilcoxon signed rank test as an alternative to the paired Student's t test since the data were not normally distributed. The protein amount was expressed as the median and interquartile range (IQR). The correlation between protein and mRNA level was evaluated by using Pearson correlation. The significance level was set at $P < 0.05$.

3. Results

The amount of NOTCH1, NOTCH3, NOTCH4 and JAG2 proteins was determined by Western blot analysis in 22 samples of endometrial adenocarcinoma and the adjacent nontumor endometrial tissue from the same patient. According to the FIGO classification, all patients had stage I endometrial adenocarcinoma. For comparison of Notch protein levels, the patients were grouped into 2 groups: stage IA (12 patients) and stage IB (10 patients).

Fig. 1A and B shows the relative expression of Notch signaling proteins in the cancer tissue in folds as compared with that of the healthy tissue.

The amount of the NOTCH1 receptor in stage I adenocarcinoma was similar to the level in the healthy tissue (median, 1.3; IQR, −1.6 to 2.2) (Fig. 1A). The median of relative NOTCH3 amount in the cancer tissue was 2-fold greater as compared with the median amount in the healthy tissue (IQR, −1.6 to 3.6) (Fig. 1A). The change in the NOTCH1 and NOTCH3 levels as compared with the amount in the control sample of the same patients was not significant. However, the difference in the amount of NOTCH4 protein between stage I adenocarcinomas and healthy tissues of the same patients was significant ($P = 0.04$) (Fig. 1A). The median of the relative NOTCH4 protein level was 1.7-folds lower in the cancer samples (IQR, −4 to 1.2).

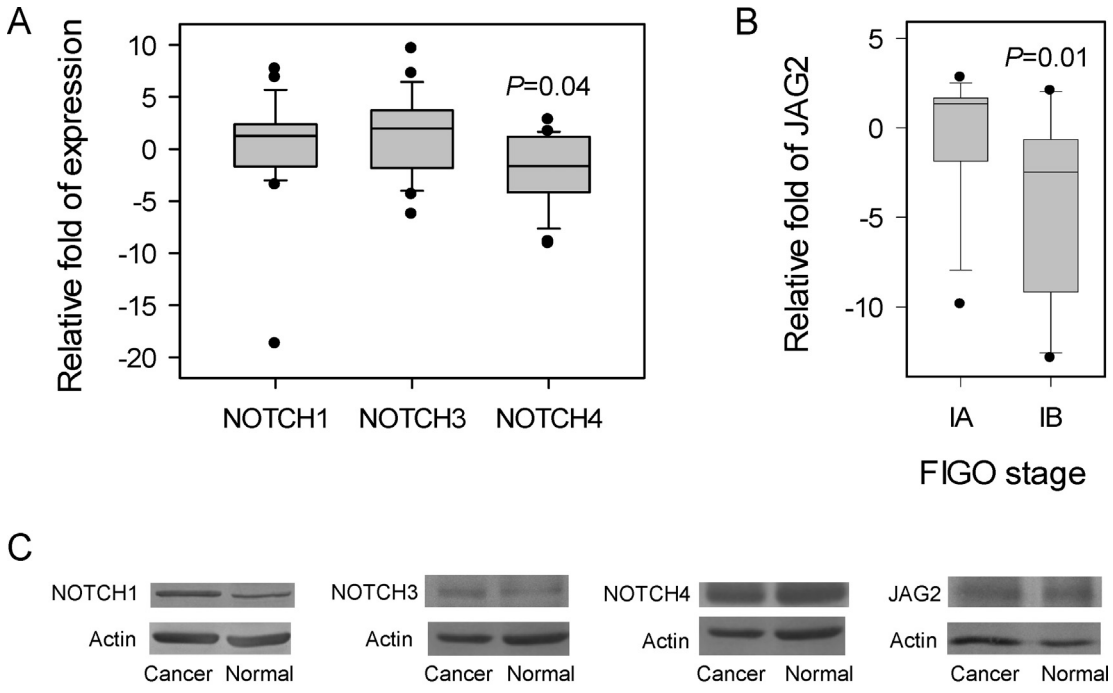

Fig. 1 – Relative expression of NOTCH1, NOTCH3, and NOTCH4 and JAG2 proteins in endometrial adenocarcinoma. Relative expression of Notch signaling pathway receptors NOTCH1, NOTCH3, and NOTCH4 (A) and ligand JAG2 (B) in stage I endometrial adenocarcinoma tissue as compared to the normal endometrium of the same patient. After densitometric analysis of the bands which was performed with ImageJ software, the amount of Notch signaling protein was normalized to β-actin. The relative expression of Notch protein was calculated as the ratio of protein amount in adenocarcinoma to that of the healthy tissue. The boundary of the box closest to zero indicates the 25th percentile, a line within the box marks the median, and the boundary of the box farthest from zero indicates the 75th percentile. The whiskers (error bars) above and below the box indicate the 90th and 10th percentiles. C, representative Western blot image of NOTCH1, NOTCH3, NOTCH4 and JAG2.

Of the 22 specimens, the relative amount of NOTCH4 protein was decreased in 13, increased in 3 samples, and unchanged in the remaining specimens. The level of Notch ligand JAG2 in stage I adenocarcinoma samples (when data of stages IA and IB were merged) was not significant, although the relative amount of JAG2 in stage IA and IB adenocarcinoma differed. There was no significant difference in the JAG2 protein level comparing stage IA cancer and healthy tissues (median, 1.3; IQR, −1.9 to 1.7). However, the difference in the amount of JAG2 between stage IB adenocarcinoma and healthy tissues was significant ($P = 0.014$) with the median being 2.5 times lower in stage IB adenocarcinoma (IQR, −9 to −0.7) (Fig. 1B). The amount of JAG2 was reduced in the majority of stage IB adenocarcinomas.

Fig. 1C shows representative Western blot in the cancer and normal tissues.

4. Discussion

The endometrium undergoes a well-coordinated and controlled process of proliferation and differentiation in premenopausal women. Under estrogen stimulation, during the proliferative phase endometrial cells are in a state of intense proliferation. Contrary, during the secretory phase, proliferation becomes insignificant while cells are induced to differentiate themselves. After menopause, the endometrium losses the functional layer [15]. The decrease of NOTCH1, NOTCH4 and JAG1 protein expression was detected in menopause [8]. Notch receptors and ligands are ubiquitous in endometrial cells and the majority of biological processes regulated by Notch signaling are closely associated with the growth and differentiation of the endometrium [16].

The role of the Notch signaling in endometrial cancer seems to be controversial. It was suggested that human NOTCH4 is involved in changes of the endometrium and also in the development of endometrial cancer [17]. Also it was shown that NOTCH1 and JAG1 increase from proliferative to secretory phase, while NOTCH4 in opposite – decreases [8]. In the pathological endometrium, an increased expression of NOTCH1 was detected from polyps to carcinoma and decrease of NOTCH4 and JAG1 was observed. However, Mitsuhashi et al. demonstrated that the expression of NOTCH1, NOTCH3, JAG1 and DLL4 proteins was higher in endometrial carcinoma and they suggested that the expression of Notch-related molecules is not directly involved in the proliferation or differentiation of cells in the normal endometrium [10]. It is important to note, that all these results were obtained, comparing protein levels in endometrial cancer versus endometria of noncancer patients.

In this study, we used a different approach – we normalized the amount of NOTCH1, NOTCH3, NOTCH4 receptors and ligand JAG2 to nontumor endometrial tissue sample of the same patient. We analyzed the samples of early stage (IA and IB) I type endometrioid adenocarcinoma patients from 22 patients, most patients were postmenopausal. The difference between stage IA and IB adenocarcinoma is that in stage IA tumor is limited to the endometrium and in stage IB, the tumor is less than or equal to half of myometrial invasion. Type I endometrioid carcinomas are estrogen-related tumors that arises in the setting of endometrial hyperplasia. They are associated with a number of well-described molecular genetic alterations and inactivation of DNA mismatch repair [15].

Our investigations showed that the level of NOTCH1 and NOTCH3 proteins in stage I endometrial adenocarcinoma were unchanged compared to adjacent non-tumor endometrial tissue. However our previous results showed a reduced amount of NOTCH1 and NOTCH3 mRNA in the endometrial cancer tissues [12]. The fact that protein level remains unchanged while mRNA decreases could be explained that the stability of Notch proteins in non-tumor tissues is lower than in tumor. It has been demonstrated that Notch activity and stability can be regulated by glycosylation and other posttranslational modifications of Notch receptors and ligands [18].

We found that relative amount of NOTCH4 was decreased in the majority of stage I adenocarcinoma samples and the difference of protein level in the samples of adenocarcinoma compared to normal tissue was statistically significant. According to the Pearson correlation test, we determined a positive correlation between mRNA, as determined in our previous study [12], and protein expression level of NOTCH4 in samples from 22 patients ($r = 0.452$, $P = 0.03$). Notch ligand JAG2 protein was unchanged in stage IA adenocarcinoma. Whereas in the majority of IB adenocarcinomas the level of JAG2 was reduced and the difference of JAG2 expression compared to normal tissue was statistically significant. These data suggest that Notch pathway has rather tumor-suppressive than oncogenic role in human endometrial cancer cells and activation of Notch signaling pathway should be evaluated as a therapeutic target.

5. Conclusions

The protein level of Notch receptor NOTCH4 and ligand JAG2 was significantly changed in stage I endometrial adenocarcinoma compared to adjacent non-tumor tissue. The reduced amounts of NOTCH4 and JAG2 proteins along with the decreased level of Notch signaling molecules coding mRNA, as reported in our previous study, supports the notion that Notch pathway has rather tumor-suppressive than oncogenic role in human endometrial cancer cells. Further detailed studies approving this hypothesis and a potential of Notch pathway activation as a therapeutic target for endometrial cancer should be evaluated.

Conflict of interests

The authors state no conflict of interests.

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