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The role of the βKlotho gene in uterine endometrial cancer

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ABSTRACT

Objectives: Endometrial cancer is the most common cancer of the female genital organs in developed countries, accounting for approximately 50% of all gynecological cancers. The Klotho gene was discovered in 1997 as an anti-aging gene that, when overexpressed, may extend the lifespan, but when disrupted, may be a factor responsible for premature aging syndrome.

The aim of the study is to assess the relationship between the clinical and pathological features of endometrial cancer and βKlotho gene expression.

Material and methods: The expression of βKlotho gene was studied in 138 cases of endometrioid endometrial carcinoma specimens using Real Time PCR reaction in RNA isolated tissue samples by commercial tests. The expression profile was correlated with the clinicopathological characteristics of endometrial carcinoma. The chi-square independence test and Fisher's test for four-field tables were used to assess the statistical significance of the observed relationships.

Results: Significant relationships were found between βKlotho gene expression and FIGO clinical stage, the degree of histological differentiation and the presence of metastases in the lymph nodes. Higher levels of gene expression correlate with lower degrees of clinical staging according to FIGO, the presence of highly-differentiated endometrial cancer (G1) and the absence of lymph node metastases.

Conclusions: The βKlotho gene expression might be involved in endometrioid endometrial cancer tumorgenesis. The βKlotho may in future be used as an useful indicator for endometrial cancer, although further studies are needed.

Key words: endometrial cancer, Beta Klotho gene

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INTRODUCTION

Malignant neoplasms are the second most frequent cause of death in Poland, being responsible for more than 26% of deaths among men and 23% among women. Endometrial cancer is the most common cancer of the female genital organs in developed countries, accounting for approximately 50% of all gynecological cancers. In Poland, in 2013, 5706 women were diagnosed with this cancer and 1243 died. The abnormal expression of Klotho proteins, as well as mutations in the genes encoding them, have been demonstrated in the case of prostate, bladder, lung or stomach cancer [1]. In recent years, it has also been proven that these disorders can play a significant role in the development of breast, ovarian and endometrial cancers.

The Klotho gene was discovered in 1997 as an anti-aging gene that, when overexpressed, may extend the life span, but when disrupted, may be a factor responsible for premature aging syndrome [2, 3]. The name of the gene and protein is derived from the Greek goddess Klotho, who spun the thread of human life and decided on its length [4, 5]. The human Klotho gene (KL) consists of five exons and four introns located on chromosome 13, and produces two transcript forms, these being known as the intraepithelial and secretory forms. The form secreted into the bloodstream, i.e. the secretory form, is created by the excision of the endothelial domain from the full transcript, i.e. the intraepithelial form, consisting of 1014 amino acids. While the membrane Klotho protein is expressed at

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a higher level in mice [6], the secretory form predominates in humans [7].

The signal sequences of the Klotho gene are found at its N-terminus, and the membrane domain and short cytoplasmic domain at the C-terminus. The Klotho part of the transmembrane segment consists of two repeats inside the gene (KL1 and KL2) that divides the homology sequence with β-glucosidase [8]. KL1 and KL2 are components of the Klotho protein structure.

In 2000, the βKlotho gene (KLB) coding for the monotopic transmembrane protein was discovered as a homologue of the αKlotho gene (αKL — Mouse Klotho α gene) of mice [5]. The *KLB* gene consists of five exons and four introns; however, while the mouse form is 35.59 kbp and located on chromosome 5, the human form measures 44.68 kbp and is found on chromosome 4. The expression profile of *KLB* is significantly different from that of the αKlotho gene: In mice, βKlotho gene expression occurs primarily in the liver and pancreas, while *KLB* expression is observed in the skin, skeletal muscle, stomach, small intestine and lung [9–11]. In humans, *KLB* expression has been detected mainly in the liver, adipose tissue and breast tissue, and to a lesser extent in the spleen, testes, prostate, eyeball and brain [12].

The transmembrane protein βKlotho has a molecular mass of 119.8 kDa and is a homologue of the αKlotho protein; in humans, the two proteins demonstrate approximately 41.2% amino acid compatibility [13], while the mouse and human forms of the β-Klotho protein share 79% similarity [14]. Three domains are distinguished in the structure of the βKlotho protein: a C-terminal extracellular domain, an intramembrane domain and a short cytoplasmic domain [15].

In addition, unlike αKlotho, previous studies have not shown that the βKlotho gene transcript undergoes an alternative assembly process that would result in the secretion of this protein. The αKlotho protein possesses a short amino acid sequence (KKRK), within which the protein is proteolytically cleaved; however, this sequence is lacking from the βKL2 region [9].

βKlotho interacts with the endocrine growth factors FGF21 and FGFR1, which transmit the biological cellular response via growth factor receptors (FGFR).

Aim of the study

The aim of the study is to determine the relationship between the clinical and pathological features of endometrial cancer and βKlotho gene expression.

The study analyses the relationship between the range of βKlotho expression and the FIGO (*Fédération Internationale de Gynécologie et d'Obstétrique*) stage, the histological differentiation and the presence of lymph node metastases in endometrial cancer.

MATERIAL AND METHODS

The design of the study was presented and approved by the Ethical Committee of the Medical University of Lodz (RNN/617/14/KB). All patients participating in the study gave their informed consent to take part. The samples of cancer tumor tissue were collected from 138 patients treated surgically in the Department of Gynecological Oncology, Medical University of Lodz for endometrioid endometrial cancer. The mode of treatment was determined following pathological examination of endometrial tissue, collected before surgery; participation in the study had no influence on therapeutic decisions. In the examined group of patients, fresh cancer tissue samples taken from the removed uterus were immediately placed in RNAlater (Ambion, USA) and stored at -80°C for subsequent RNA extraction and testing.

Gene expression analysis was carried out as follows.

RNA isolation

The fragmented tissue samples were suspended in 500 µl buffer containing 10 mM NaCl, 500 mM Tris (pH 7.6), 20 mM EDTA, 1% SDS and 500 µl/mL proteinase K and incubated at 50°C for eight hours. The RNA was then isolated from the lysates obtained using 500 µl of TRI Reagent: TRI (Total RNA Isolation) is a mixture of phenol, guanidine isothiocyanate and other compounds causing cell lysis and inactivation of endogenous RNases. After 10 minutes of incubation at room temperature, 200 µl of chloroform was added and mixed for 30 s using a shaker. The samples were then centrifuged at 4°C for 15 minutes at 12,000 x g. The aqueous phase was transferred to a new tube, 500 µl isopropanol was then added and the contents of the tube were mixed. After 15 minutes of incubation at room temperature, the samples were centrifuged at 4°C for 10 minutes at 12,000 xg. The supernatant was removed and 1 mL of 75% ethanol was added to the pellet of RNA. Samples were then centrifuged at 4°C for five minutes at 12,000 x g. After removal of the supernatant, the pellet was dried at room temperature for five minutes. The pellet was resuspended in 20 µl of water lacking ribonuclease activity using DEPC. RNA samples were stored at -20°C. Spectrophotometric analysis was then performed to evaluate the purity and concentration of RNA and DNA.

The purity of the obtained RNA preparations was determined spectrophotometrically by measuring the absorbance of each sample at 260 nm and 280 nm. The accepted criterion for DNA and RNA purity was the value A260/A280, which was within 1.8–2.0. The RNA concentration was determined by measuring the absorbance at 260 nm. This value corresponds to the following relationship:

- 1 OD = 40 µg RNA/mL,
- analysis of βKlotho gene expression at the mRNA level,
- reverse transcription reaction.

The reverse transcription reaction was performed using the High Capacity cDNA Reverse Transcription Kit according to the manufacturer's instructions. First, 2 µg of total RNA (1 µg/µl) was suspended in a 20 µl mixture containing 2 µl of 10 × RT Buffer, 0.8 µl dNTP (100 µM), 2 µl of 10 × RT Random Primers, 1 µl of MultiScribe Reverse Transcriptase, 1 µl RNase inhibitor, 11.2 µl of DEPC treated water. The prepared samples were incubated for 10 minutes at 25°C, followed by 120 minutes at 37°C and 5 minutes at 85°C. The obtained cDNA was stored at -20°C.

PCR reaction with real-time product volume increase analysis (Real-Time PCR)

The Real-Time PCR (polymerase chain reaction) reaction allows the number of copies of the mRNA of a particular gene to be determined in an original sample using gene-specific probes labeled with fluorescent dyes. The reaction contained 0.5 µl of cDNA, 0.5 µl of 20 x TaqMan® Gene Expression Assays, 5 µl TaqMan® Universal PCR MasterMix containing TaqMan® DNA polymerase, dNTP, reaction buffer and 4 µl of water. The Real Time PCR reaction was carried out in the Mastercycler® ep realplex thermal cycler according to the following program: pre-denaturation at 95°C for 10 minutes followed by 50 cycles of incubation at 95°C for 15 s and at 60°C for one minute.

The standard commercial probe and primer sets were used in the Real Time PCR reaction. The obtained Ct values were converted into the number of mRNA copies of the βKlotho gene per 1000 copies of *HPRT1* mRNA according to the following relationships:

- ΔCt = Ct of the test gene - Ct of the reference gene,
- L = 1000 2-ΔCt,

(where: L — number of mRNA copies of the tested gene / 1,000 copies of the mRNA of the reference gene).

Statistical methods

A statistical analysis was performed of the clinical and pathological parameters of the examined material. The Chi-square independence test was used to assess the statistical significance of the observed relationships. In cases where the assumptions for the said test were not met (e.g. fewer than five examples of any one field), Fisher's test for four-field tables was used. A value of $p < 0.05$ was considered statistically significant

Continuous variables were coded into qualitative variables based on quartile values before analysis using the chi-square or Fisher's test. As the therapeutic process had no effect in some cases, the total number of patients included in the individual calculations may be smaller than the total number of patients enrolled in the study. Patients who underwent surgery without lymphadenectomy are not included in the calculations, including the assessment of the dependence of the expression of the examined genes on the metastases of cancer to the lymph nodes. Statistical calculations and graphs were created using STATISTICA12 software (StatSoft).

RESULTS

For statistical analysis, βKlotho gene expression was divided into five categories. Category 1 consisted of cases for which the expression was absent. For non-zero values of gene expression, the values of subsequent quartiles were calculated. Category 2 included cases for which the expression value was no higher than the first quartile value. Category 3 included cases between the first quartile and the median. Category 4 included cases between the median value and the third quartile value. Any case where expression was greater than the third quartile was classified as category 5. The exact assignment of individual values to categories is provided in Table 1.

The analysis was started by examining the relationship between the range of βKlotho expression values and FIGO clinical stage.

The relationship between the range of βKlotho expression values and the FIGO clinical stage

The analysis indicates that higher FIGO stages (i.e. 3 or 4) occur almost exclusively when βKlotho gene expression was absent (see Tab. 2). When βKlotho expression was present, stage FIGO1 (around 90%) was found to predominate. A statistically significant relationship was found between the occurrence of βKlotho gene expression and the clinical stage of FIGO (chi2 = 44.35, $p < 0.001$).

Table 1. Values of βKlotho gene expression and coding adopted for statistical analysis

βKlotho code	βKlotho gene expression value	Number of cases	[%] of cases
1	0	64	46.4%
2	2.13–19.76	19	13.8%
3	21.07–47.13	18	13.0%
4	47.16–79.35	18	13.0%
5	80.26–118.2	19	13.8%
Total	**0–118.2**	**138**	**100%**

Table 2. The relationship between the expression of the βKlotho gene and the clinical stage of FIGO

Expression of the βKlotho gene	FIGO: 1A, 1B, 1C	FIGO: 2, 2A, 2B, 3, 3A, 3B, 3C, 4B	Total
0	24 (38.1%)	39 (61.9%)	63 (100%)
2.13–118.2	66 (90.4%)	7 (9.6%)	73 (100%)
Total	**90 (66.2%)**	**46 (33.8%)**	**136 (100%)**
Statistical analysis	**$Chi^2 = 44.35$; $p = 0.0000$**		

The relationship between the range of βKlotho expression values and the degree of histological differentiation

βKlotho gene expression was absent in all tumors at stage G3 (grading, the degree of histological differentiation of malignant tumors). At histological stage G2, while most cases demonstrated no expression, various levels of expression were also observed. However, at stage G1, the majority of cases demonstrated high gene expression, placing them in category 5 (80.26–118.2). A statistically significant relationship was hence observed between the occurrence of βKlotho gene expression and the degree of histological differentiation (T. Fisher, $p < 0.001$, see Fig. 1 and Tab. 3).

The relationship between the range of βKlotho expression and the presence of lymph node metastases

An analysis of lymph node status and βKlotho gene expression indicates that the lymph nodes are involved primarily when βKlotho gene expression is absent.

A statistically significant relationship was found between the occurrence of βKlotho expression and the presence of metastases in the lymph nodes (T. Fisher, $p < 0.001$, Tab. 4).

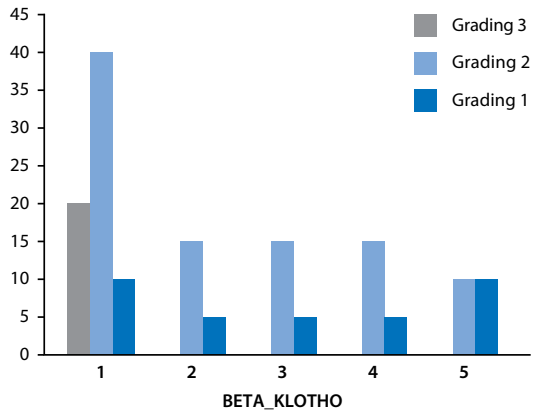

Figure 1. Relationship between the range of βKlotho expression values and the degree of histological differentiation; N — number of samples

Table 3. The expression of the βKlotho gene according to the degree of histological maturity

Expression of the βKlotho gene	G1, G2	G3	Total
0	43 (68.3%)	20 (31.8%)	63 (100%)
2.13–118.2	73 (100%)	0 (0%)	73 (100%)
Total	**116 (85.3%)**	**20 (14.7%)**	**136 (100%)**
Statistical analysis	**Fisher's test; p = 0.0000**		

Table 4. Relationship between the expression of the βKlotho gene and lymph node status

Expression of the βKlotho gene	Lymph nodes: not involved	Lymph nodes: metastatic	Total
0	42 (73.7%)	15 (26.3%)	57 (100%)
2.13–118.2	65 (97.0%)	2 (3.0%)	67 (100%)
Total	**107 (86.3%)**	**17 (13.7%)**	**124 (100%)**
Statistical analysis	**T. Fisher; p = 0,0002**		

DISCUSSION

Interest is growing in the genetic aspects of the epigenesis of malignant tumors. The life cycle of the cell is controlled at the genetic level. Hence, the genetic mechanisms responsible for carcinogenesis are typically identified according to the general scheme of carcinogenesis, from the initiation of a genetic error, through its promotion and progression, to the eventual halting of its fixation by apoptosis.

The Klotho gene has been labelled the youth gene [2, 3]. Its role in the neoplastic process has been studied in numerous cancer processes. Immunohistochemical analysis has revealed high expression of Klotho in healthy breast tissue and very low expression in breast cancer tissue. In breast cancer tissue samples, high Klotho expression was correlated with a smaller tumor size and less mitotic activity. Cervical cancer studies have found Klotho mRNA to be absent in numerous samples of cancer tissue at high stages of clinical FIGO, but not in cases of early pre-invasive oncogenesis (*carcinoma in situ*); it has also been reported that Klotho expression returns to normal after treatment, indicating that significant reduction of Klotho expression may be associated with the presence of cervical cancer. Similar results have also been observed in the oncogenesis of colon [16], stomach [17] and bladder [18] cancer.

Subsequent studies have found the Klotho gene to play a suppressing role in the neoplastic transformation of bladder cancer [18].

The βKlotho gene has been an object of interest for many scientists [19]. Initial studies examined its role in the development of liver cancer but with varying results. However, most studies suggest that βKlotho inhibits the proliferation of liver cancer cells. Poh et al. [20] report increased expression of βKlotho and FGFR4 in human liver cancer cells and in HCC (hepatocellular carcinoma) cell lines, suggesting that increased expression of KLB is associated with neoplastic transformation and the progression of liver cancer. However, other research groups have reported reduced KLB expression in HCC cell lines [20–22], and that restoration of normal KLB expression inhibited HCC proliferation.

Until now, marked KLB expression has been observed in various healthy and cancerous tissues. KLB expression is absent in the case of endometrial cancer, or at least significantly lower than in healthy endometrial tissue [22]. The literature concerning relation between βKlotho and endometrial cancer is limited. Available data suggest decreased expression of KLB in endometrial carcinomas [23].

The data from our study clearly indicates that βKlotho is involved in the process of neoplastic transformation of endometrial mucosa, and that further analysis in this area is called for. Our findings represent a more thorough assessment of the activity of βKlotho with regard to clinical FIGO staging, lymph node infiltration and tumor malignancy grade.

The higher clinical FIGO stages, i.e. 3 or 4, were found to be characterised almost exclusively by absent expression of the βKlotho gene (Tab. 2). When expression was present, approximately 90% of cases were observed in clinical stage 1. Moreover, βKlotho gene expression was always found to be absent in samples of histological grade G3, while it tended to be very low or zero for grade G2. In contrast, gene expression reached category 5 in our study for grade G1. In addition, lymph node involvement was typically associated with absent βKlotho gene expression, with most samples expressing βKlotho also demonstrating lymph nodes free of metastases. Statistical analysis showed a strong dependence of βKlotho expression on FIGO clinical stage, histological malignancy grade and presence of infiltrated lymph nodes. Our findings suggest that a lack of βKlotho gene expression promotes the development of neoplastic processes in endometrial cancer, while high βKlotho gene expression may act to suppress neoplastic transformation.

CONCLUSIONS

Our results support the hypothesis indicating the role of βKlotho gene expression in endometrioid endometrial cancer pathogenesis. However further studies in this field are needed to elucidate suggested relations.

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