P R A C E O R Y G I N A L N E
ginekologia

Aberrant claudin-4 transcript levels in eutopic endometrium of women with idiopathic infertility and minimal endometriosis

Ocena poziomu transkrypcji klaudyny-4 w eutopowym endometrium kobiet z niepłodnością pierwotną i minimalną endometriozą

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Abstract

Introduction: *Claudin-4 (CLDN4) is a transmembrane protein, responsible for cellular contact and organization. A different expression of claudin 4 in the endometrium, depending on the menstrual cycle and with peak at the aim of the 'implantation window', has been observed. CLDN4 is believed to play an important role in embryo implantation.*

The aim: *The aim of the study was to compare the mRNA CLDN4 expression levels in two subgroups of infertile women (idiopathic infertility or minimal endometriosis) and compare them to fertile controls.*

Method: *The study included 36 women with idiopathic infertility and 24 with minimal endometriosis. The control group comprised 26 women. Eutopic endometrium samples were collected with a Pipelle device during the implantation window. Firstly, mRNA was extracted from the endometrium and reverse transcribed into cDNA. Real time PCR was used for the assessment of relative expression levels.*

Results: *The observed transcription level of CLDN4 did not differ statistically between the studied groups, but was significantly higher when compared to controls.*

Conclusions: *Exceedingly high levels of CLDN4 might negatively influence fertility rates.*

Key words: **claudin** / **endometrium** / **idiopathic infertility** / **endometriosis** /

Streszczenie

Wstęp: *Klaudyna 4 (CLDN4) jest białkiem transbłonowym, odpowiedzialnym m.in. za wzajemny kontakt komórek i ich organizację. W endometrium zaobserwowano zmienne natężenie ekspresji CLDN4, zależne od fazy cyklu, z maksimum w „oknie implantacyjnym". Zakłada się, że CLDN4 może odgrywać istotną rolę w procesie implantacji zarodka.*

Cel pracy: *Celem pracy było porównanie poziomu mRNA CLDN4 w grupach kobiet niepłodnych z niepłodnością idiopatyczną i endometriozą minimalnego stopnia w odniesieniu do grupy kontrolnej.*

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Metoda: *Plan badań uzyskał zgodę komisji bioetycznej. Do badań zakwalifikowano 36 kobiet z niepłodnością idiopatyczną, 24 z kobiety endometriozą minimalnego stopnia. Grupa kontrolna obejmowała 26 kobiet.*

Materiał badawczy stanowiło eutopowe endometrium, pobrane pipellą w oknie implantacyjnym. Z bioptatów wyizolowano RNA. cDNA uzyskano przy pomocy odwrotnej transkrypcji. Do wyznaczenia względnego poziomu transkrypcji zastosowano metodę real-time PCR.

Wyniki: *Poziom transkryptu CLDN4 nie różnił się w sposób istotny statystycznie pomiędzy grupami badanymi, ale był istotnie statystycznie wyższy w obu grupach badanych w odniesieniu do grupy kontrolnej.*

Wniosek: *Zbyt wysoki poziom ekspresji CLDN4 może mieć związek z zaburzonym rozrodem.*

Słowa kluczowe: **klaudyna** / **endometrium** / **niepłodność idiopatyczna** / **endometrioza** /

Introduction

Embryo implantation depends on a close interaction between the embryo and the endometrium [1]. Faulty implantation might lead to many problems including infertility, miscarriage, IUGR and hypertension in pregnancy [2, 3, 4]. Despite diagnostic advances in the reproductive field, the cause of infertility continues to be idiopathic in about 10% of couples [5]. Also, the reasons behind infertility in patients with minimal endometriosis remain to be elucidated [6]. Proper early dialogue between the embryo and the endometrium warrants successful pregnancy.

While great strides have been made to detect poor quality of the embryo, either by morphologic or by genetic indices, the endometrial part of receptivity remains poorly understood [7]. Thanks to recent advances in genetic technologies, such as gene chip matrices, we are now able to compare thousands of genes simultaneously between various times of the endometrial cycle [8]. Pinpointing the 'implantation window', that is the period of maximal endometrial receptivity, allows for precise comparison of up- and down-regulated genes, that might prove indispensable for successful implantation to occur.

Claudin-4 (CLDN4) is one of the genes that were found to be significantly up-regulated during the period of endometrial receptivity. The first paper describing the up-regulation of claudin-4 was the work of Kao et al. [9] Later their results were confirmed by two independent researchers, namely Rijsjewik and Giudice [10, 11]. Claudin-4 is an integral membrane protein and a member of a large family of transmembrane tissue-specific proteins, that are essential components of the intercellular tight junction and regulate paracellular ion flow and cell polarity [12]. Claudin-4 is found on nearly all epithelial and endothelial cells, including endometrial tissue. As such, it might contribute significantly to establishing the connection between the embryo and the endometrial surface, as well as play a role in cell-to-cell signaling pathways [9, 10, 13]. In humans, claudin-4 was first described by Katahira [14]. The aberrant expression of claudin-4 was noted in various cancers [15, 16, 17, 18, 19, 20, 21, 22]. Recently, its role in the pathogenesis of endometriosis has been brought to attention [23, 24].

Therefore, we decided to test the claudin-4 mRNA expression levels in women with idiopathic infertility and infertile women with minimal endometriosis and compare those results with endometrial expression levels of claudin-4 of women with proven fertility.

Materials and methods

Patients

The study was conducted at the Division of Reproduction, Department of Obstetrics and Gynecology, Poznan University of Medical Sciences, Poland.

Only those infertile patients in whom either all diagnostic tests were negative or those patients who presented only with minimal endometriosis were included in the study. The following tests were performed in each couple: semen analysis, ovulation tracking, hysterosalpingography, hormone studies and laparoscopy with hysteroscopy. We gathered 36 idiopathic infertility patients and 24 infertile patients with minimal endometriosis, as assessed by laparoscopy and histology, according to the American Fertility Society [25].

Mean duration of infertility in each group was 3.4 years (1-5 years) and 3.2 years (1.2-6 years), respectively. Also, 26 patients, matched for age, with at least one child, no negative history of infertility and endometriosis, no miscarriages, were enrolled in the study as controls. Those patients were admitted to the hospital for non-endometrial diseases, and were approached to donate the endometrium. The study protocol was approved by the local ethical committee, and the patients signed an informed consent form. None of the patients in the study and the control groups had taken any hormonal preparations for at least three months prior to the study.

Collection of samples and RNA isolation

All patients from the study and control groups had a biopsy sample taken 7-9 days after the ovulation, confirmed by ultrasound follicular tracking. The endometrial sample was placed in RNAlaterTM buffer from Qiagen (Hilden, Germany) and frozen till extraction.

The isolation of total RNA was done with the use of RNAeasy Mini Kit (Qiagen, Germany). QiaShredder columns (Qiagen) were used for homogenization according to the manufacturers instructions. The total mRNA was treated with QantiTect Reverse Transcription (Qiagen) to acquire cDNA.

Primers design and qPCR reaction

RNA specific primers for RealTime PCR were created with the Primer3 software (http://frodo.wi.mit.edu/primer3) based on the mRNA (ENSG00000189143) sequence from ENSEMBL database (http://www.ensembl.org/index.html). The

thermodynamic features of designed primers were first checked in OligoAnalyzer1.2 software, next specificity of constructed primers was checked against BLAST database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The following primers were constructed: GAPDH - forward: ACAGTCAGCCGCATCTTCTT and reverse: ACGACCAAATCCGTTGACTC and for claudin 4 - forward: TCTGTCTGCCTGCATCTC and reverse: AAGGCCTCAGCCATACTC.

The resulting 103bp cDNA product was checked on agarose gel to confirm the specificity of primer sets. Additionally, second derivative analysis melting curve product in Real Time PCR reaction of these primer sets was used to confirm the specificity. The resulting cDNA samples were used as the matrix for RealTime PCR which was conducted in duplicate, on RotorGene 3000 RealTime thermocycler (Corbett Research). The optimized protocol was used with the mastermix including HotStart polymerase (DyNAmo HS SYBRGreen qPCR Kit from Finnzymes, Espoo, Finland). The thermal profile was as follows: denaturation at 95^0C, 15min; amplification and quantification at 94^0C for 10sec, followed by 55°C for 25sec and finally 72^0C for 30sec; followed by first data acquisition at 79^0C. The thermocycler was set to 40 runs. After that process 72^0C, 10min was run and next melting curve was run, from 72^0C to 95^0C, rising by 0.5^0C with each step with continuous fluorescence measurement. The expression of claudin-4 was established according to the reference gene, namely glyceraldehyde-3-phosphate dehydrogenase (GAPDH), whose expression in cells is universally considered as constant across the menstrual cycle. The mean GAPDH expression levels did not differ significantly among the groups.

Statistical analysis.

For statistical analysis, SigmaStat3.5 software was used. The analysis of the results was based on the Kruskal-Wallis One Way Analysis of Variance on Ranks assessment; $p < 0.05$ was considered statistically significant.

Results

We observed statistically significant differences in CLDN4 relative transcript levels in eutopic endometria between controls and patients with idiopathic infertility and between controls and women with minimal endometriosis. The relative levels of claudin mRNA expression were statistically significantly lower in fertile controls when compared to the studied groups. However, there was no difference in the CLDN4 relative transcription levels between both groups of infertile patients (idiopathic infertility and endometriosis). The results are presented in Table I and scatter diagram, Figure 1.

Table I. Target mRNA levels were corrected to the amount of cDNA and expressed as multiplicity of these cDNA copies in the calibrator. The obtained results were compared against the control group using the Mann-Whitney Rank Sum Test.

Group	n	median	min.-max. value of CLDN4 relative transcript level	P
Healthy controls	26	63.35	2.63-650	-
Women with idiopathic infertility	36	164.0	0.46-828	0.027
Women with minimal endometriosis	24	192.5	11.3-1090	0.025

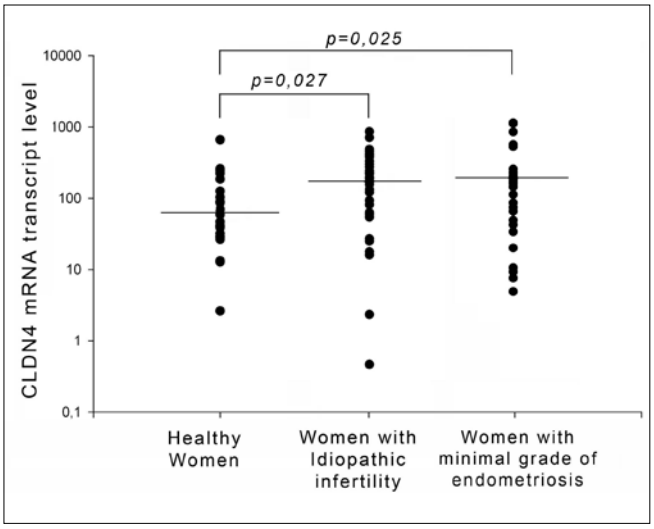

Figure 1. Relative transcript level CLDN4 in the endometrium of women from the studied and control groups. To normalize the quantity of transcripts in each sample, CLDN4 mRNA levels were normalized to the amount of GAPDH cDNA. Horizontal lines correspond to the values of medians.

Discussion

Infertility is a disease that affects many couples trying to conceive a child. The natural fecundability in a monthly cycle is estimated to be around 20-33% [26]. Part of the blame for such low chances for successful pregnancy might be attributed to embryo defects, like aneuploidy, found especially in older women [27, 28, 29].

Advances in Assisted Reproductive Technology (ART) allowed clinicians to assess embryo quality and its future potential for implantation by means of grading its morphology, fragmentation status, and recently also its genetic makeup owing to Preimplantation Genetic Diagnosis [30-32]. However, even with such sophisticated technologies, the clinical pregnancy rate remains at a disappointing 30% level at the best IVF clinics [33]. Therefore, it is clear that the endometrium plays an important role in implantation. Understanding the mechanisms behind the implantation and the ways to control it would translate into better outcomes for patients. About 40% of patients with endometriosis are infertile. The reasons for infertility in high grade endometriosis are well understood, while the mechanisms behind infertility in minimal endometriosis, without the presence of adhesions or endometriomas, are less clear [34]. Numerous factors, including hormones, antibodies and changed immunologic status, are speculated to contribute to infertility of women with endometriosis [35, 36]. Recently, the role of intrinsic changes within the eutopic endometrium of women with endometriosis has been brought up by some researchers [37]. Whether these changes are the cause or effect of endometriosis, and whether they could be considered to be causative agents for infertility that often accompanies this disease, remains to be elucidated.

Up till now, the search for the so-called markers of endometrial receptivity has relied on studying single factors or family of genes. Gene matrix analysis allowed studying thousands of genes at a single pass. This in turn allowed pinpointing candidates for markers of endometrial receptivity. Some of these up- and down regulated genes might be indispensable for the implantation process, while others might turn out to be redundant.

To the best of our knowledge, this paper is the first attempt to elucidate the potential role of claudin 4 in reproduction in natural cycles. We selected women with idiopathic infertility and infertile women with endometriosis as the potential candidates for, as yet undiscovered, errors in genetic code that might lead to infertility. We hypothesized that some cases of infertility in those patients might be attributed to aberrant expression of claudin 4 mRNA in the endometrial tissue.

The role of claudin-4 in reproduction is not well understood. Studies from Husjewic assessed the role of claudin-4 in ion transport through the pores in tight junctions between cells, favoring chloride influx, while blocking the influx of sodium ions [5]. With the initial attachment of the embryo to the endometrial surface and further deeper invasions, the environment between them emerges as a major contributor to either pregnancy success or failure. Since numerous studies proved that claudin mRNA expression is upregulated during the midluteal phase of the endometrial cycle, one might speculate that the function of claudin-4 during the implantation process might be an important one [9, 10].

We found statistically significant differences in claudin-4 mRNA expression levels between the studied groups and fertile controls. Patients with minimal endometriosis often suffer from infertility. Some studies suggested that eutopic endometrial samples from women with endometriosis differ markedly from eutopic endometria obtained from women without this disease. Authors of these studies showed that apoptosis, MMP 3, MMP7 are differently regulated within the eutopic endometrium of women with and without endometriosis [38, 39, 40, 41].

Contrary to recently published study from Pan et al., who found no significant differences in eutopic endometrium between the studied groups, both at the mRNA and protein level, we found higher levels of mRNA expression in the eutopic endometrium of women diagnosed with minimal endometriosis when compared to fertile controls [24]. These authors however, also found that ectopic endometrial lesions had significantly down-regulated claudin-4 expression when compared to eutopic endometrium, which might suggest that an altered environment of the peritoneal cavity in women with endometriosis could 'transform' the biochemical and genetic pathways of shed eutopic endometrial cells, allowing them to grow and function as endometriosis implants. On the basis of the result of our study we might speculate, that the up-regulation in claudin expression is an intrinsic factor that is already present in the eutopic endometrium. These changes might prove responsible for the survival and invasive potential of endometrial cells found in women with endometriosis.

We also found a higher expression of claudin-4 mRNA in the group of patients with idiopathic infertility when compared to fertile controls. Women with idiopathic infertility are particularly difficult to treat as all the treatments offered are empirical as best. Those patients are ideal candidates for studies of, as yet undiscovered, causes of human infertility. Taking into consideration the results of our investigation, it seems safe to conclude that aberrant expression might be a common cause of infertility in selected group of patients.

We cannot rule out that patients with idiopathic infertility might have microscopic lesions of endometriosis, therefore some of them might exhibit the same changes in claudin expression in the eutopic endometrium as women with endometriosis.

Our results are confirmed by the study of Serafini et al., who assessed the chances of conception and pregnancy in women undergoing IVF programs [42]. They proved that women with low claudin-4 in a midluteal biopsy taken in a cycle preceding the IVF treatment, had the highest chances of successful implantation and pregnancy. The results were similar even after correction for differences of age. The question remains whether that could be viewed as a proof of detrimental role of claudin-4 in the implantation process and, if so, why the gene matrix studies revealed strong up-regulation of the claudin-4 genes during the implantation window. There is accumulating evidence that IVF treatment influences a number of factors within the endometrium, at the protein, receptor and genetic levels [16].

Since the collection of samples took place in a non-conceptive cycle, one cannot draw conclusions as to the effects of powerful hormonal stimulation used for controlled ovarian hyperstimulation protocols on the expression of different markers of implantation. It could be speculated that hormonal treatments have selectively 'corrected' the low expression of claudin-4 in those patients, resulting in better pregnancy rates compared to women with 'normal' claudin-4 levels. There are also reports that link progesterone levels to higher claudin-4 expression [43].

In a cited study, all women received 1200 mg daily doses of micronized progesterone, and that treatment could influence or 'rescue' claudin expression, resulting in improved pregnancy rates. Such positive response to progestin supplementation, namely increased expression of claudin 4 and better pregnancy rates, might explain, on the molecular level, why some patients might benefit from this kind of therapy.

Conclusion

In conclusion, it seems that exceedingly high levels of claudin expression in the eutopic endometrium play a role in infertility in both women with minimal endometriosis and idiopathic infertility. The role of claudin in the development of endometriosis remains to be elucidated.

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