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ORIGINAL ARTICLE

Differential expression of Claudin-3 and Claudin-4 in endometriosis

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ABSTRACT
Endometriosis is a chronic inflammatory disease that is characterized by presence of endometrial lesions in extra-uterine tissues, affecting ~ 190 million women globally. Our study investigated epithelial mesenchymal transition in different grades of endometriosis by tracking the changes as we move from grade 1 to grade 4. Furthermore we also explored the role of claudin-3 and claudin-4 in different grades of endometriosis. We found decreased expression of claudin-3 and claudin-4 in different grades of endometriosis when compared with the control group. Significant decrease in expression of claudin-3 and claudin-4 was evident in grade 3 and grade 4 endometriosis than in grade 1 and grade 2 endometriosis. Claudin being an integral part of tight junctions is inevitable for maintaining cell-cell integrity and epithelial homeostasis. Undermining the claudin-3 and claudin-4 function is assumed to play an important role in pathogenesis of endometriosis because disruption of tight junctions would set free endometrial cells for migration and invasion. Comprehending the role of claudin-3 and claudin-4 would unveil new insights into underlying molecular mechanisms in endometriosis and it also creates opportunities for designing new therapeutic approaches to treat the disease.
Keywords*: Endometriosis, Epithelial-Mesenchymal Transition (EMT), Claudin-3, Claudin-4, Tight junctions (TJ), Flow cytometry*

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INTRODUCTION

Endometriosis is a disease characterized by the presence of endometrial glands and stroma outside the uterine cavity and pelvic compartment. It is an chronic inflammatory condition, affecting women mostly in their reproductive age, associated with symptoms such as pelvic pain, painful intercourse, painful urination, painful bowel movements, infertility, abdominal bloating, nausea, fatigue, depression, and anxiety [1]. Globally endometriosis affects ~5% (190 million) of women population, who falls predominantly between 25 years and 35 years of age [2, 3]. Despite the fact that there is no cure for the disease, symptomatic treatment can be done using medicines and if demands surgery becomes inevitable in critical situations. Interestingly, even after extensive research and investigation the pathogenesis of endometriosis is not properly understood and is entangled in several controversies. Among the several theories proposed from time to time, the most widely accepted one is the implantation theory [4]. It is based on the assumption that a small and early lesion is implanted on the extra-uterine or pelvic cavity tissue foundation being laid by uterine endometrial cells transported via retrograde menstruation; and later lesions grows and invades leading to pathogenesis. In addition, presence of lesions at more distant locations such as thoracic or cerebellar endometriosis [5-7] might be due to the dispersion of endometrial cells through the lymphatic systems [8, 9]. Implantation theory necessitates several criteria to be met: i) retrograde menstruation should happen [10-12]; ii) retrograde refluxed menstrual efflux must have viable endometrial cells [13, 14]; and iii) shed endometrial cells should retain adhesive capacity so that they attach, proliferate, and differentiate onto the peritoneum [15]. This detachment of uterine endometrial cells

and its attachment into the extra uterine tissues requires a mechanism known as Epithelial-mesenchymal transition (EMT), making the EMT integral part of implantation theory.

An epithelial-mesenchymal transition (EMT) is an orchestrated biological process that involves transformation of polarized epithelial cell (that are adhered to basement membrane via its basal surface) to mesenchymal cell phenotype, that possess distinct features such as increased migratory aptitude and invasiveness, elevated resistance to apoptosis, and escalated production of Extracellular Cellular Matrix (ECM) components [16]. Completion of EMT is coupled with several changes such as degradation of basement membrane, formation of mesenchymal cells, loss of cell polarity, disruption of cell-cell junctions, and reorganization of ECM [17, 18]. Though EMT is indispensable for embryonic development, if deregulated it unleashes opportunity for several diseases [19].

As epithelial cell-cell contact is disrupted in EMT, several researchers extensively studied the role of tight junctions (TJ) in endometriosis. Tight junctions are macro protein complexes that encompass 40 different proteins, among which claudin proteins play a pivotal role. Claudin, a transmembrane protein, is a critical component of a Tight Junctions strand and so far 27 members of claudin family were identified [20-22]. Claudin play crucial role in maintaining cell-cell integrity, regulating paracellular permeability and maintaining cell polarity in epithelial and endothelial cell sheets. Deregulation of claudin is implicated in several diseases ranging from manageable endometriosis to devastating cancers. The role of claudin-7 is investigated in human HCC827 lung cancer cells, as claudin-7 modules cell adhesion, migration and invasion [23]. Furthermore, aberrant expression of several Claudin namely: claudin-18 role in gastric, pancreatic, and biliary cancers [24-26] and claudin-1 role in colorectal cancers, and neck and squamous cancers is also revealed [27, 28]. In addition, claudin that has potential barrier function if deregulated also open gates for several intestinal disorders [29].

Role of claudin in endometriosis has been investigated by several researchers previously. A study reported increased expression of claudin-10 in endometriosis and its mislocalization in ectopic endometriosis [30]. Gaetje R *et al* reported reduced expression of claudin-3, claudin-4 and claudin-7 in ectopic endometrium and suggested that down regulation of these claudins might be contributing to endometriosis, mediated by endometrial cell detachment and invasion into pelvic organs [31]. Improper localization of claudin-11 in ectopic endometrium is also assumed to contribute to endometriosis [32]. Though few studies reported the role of claudin-3 and claudin-4 in endometriosis, there are also studies that contradicted the observations; and on the other hand these studies have limitation of small sample size.

So, our study aimed to get a wider and clear picture regarding the role of claudin-3 and claudin-4 in endometriosis by taking a large sample size. Furthermore, claudin-3 and claudin-4 roles in endometriosis are not studied in the Indian population. As genotype variability cannot be ignored, we aim to investigate the role of claudin-3 and claudin-4 in endometriosis in the Indian population so that molecular pathways deciphered can be exploited in designing therapeutic approaches. Moreover, earlier studies focused more on advanced stages of endometriosis (grade 3 and grade 4) without showing dissimilarities in endometrial cells from nascent (grade 1) to end stage (grade 4). So we also attempted to understand this journey so that it might open a window for new dimension in treating disease.

MATERIAL AND METHODS

Selection of Subjects

All the subjects were enrolled from the Department of Obstetrics and Gynecology, MHRT Hospital & Research Centre, Hyderabad, Telangana, India. Subjects were clinically diagnosed and confirmed for the presence of endometriosis lesions investigated using laparoscopy and histo-pathological analysis. Further, the grading of these endometriosis lesions was performed as per the guidelines of the American Society of Reproductive Medicine Revised System. The study protocols were approved by the Institutional Ethics Committee, MHRT Hospital & Research Centre, Hyderabad.

Study population

A total of 250 women were enrolled in this study, of which 50 were controls and 150 were endometriosis patients with different grades. Control group included women who underwent sterilization for family planning and tubectomy, totally free of any pathology. All study participants underwent laparoscopy for various complications such as pelvic masses, pelvic pain, infertility, and uterine leiomyoma. After laparoscopy, a thorough inspection of the abdominopelvic cavity was performed to detect any typical or atypical endometriotic implant and scaring, and all possible endometriotic lesions were excised and sent for pathological examination. Women were assigned to different endometriosis groups (Grade 1, 2, 3 and 4) or endometriosis-free control groups based on pathological reports. Women with postmenopausal status, previous hormonal use within three months, adenomyosis or malignancy were excluded from this

study. Of the total 200 women identified with endometriosis, all the grades (1, 2, 3 and 4) had 50 members each.

Sample collection

Clinically confirmed endometriosis tissue biopsies were collected and placed in 1 mL of normal saline during laparoscopy. In addition, biopsy tissues were collected from endometriosis-free women subjects, who were taken as a control group. Informed written consent was taken from all the subjects prior to the collection of tissue biopsy samples. All the tissue biopsies were transported to a sample processing laboratory in aseptic conditions and tissues were washed twice with normal saline. Cells and mRNAs are isolated from the tissues to perform the experiments.

Cell isolation and enrichment from endometrial tissue biopsies

Tissue biopsies from each grade were digested for 30 min using collagenase and later digested tissues were passed through 0.44 µm sieve to get single cells. The isolated single cells were placed in DMEM/F12 (Gibco™ 31885049, Thermo Fisher Scientific) culture media that is supplemented with 10% FBS (Gibco® Qualified FCS, 26140087,Thermo Fisher Scientific), Antibiotics (Catalog #: 15240062, Thermo Fisher Scientific) in a 30 mm dish. All culture dishes were incubated in CO2 incubator with 5% CO2 at 37°C and 100% humidity. All the cultures were maintained for 5 days and morphological observations were carried out at regular intervals using microscope. Cells from each grade were trypsinized and used for Immunophenotypic characterization.

Immunophenotypic characterization

Immunophenotypic characterization of enriched cells from endometrial tissue biopsies was done through flow cytometry through the assessment of stromal/mesenchymal positive markers CD73 (BD-Biosciences), CD90 (BD-Biosciences) and CD105 (BD-Biosciences). Staining of each antibody was performed as per the manufacturer's instructions and the stained cells were assessed by cell quest software using software using BD-Via flow cytometry.

Relative expression analysis of Claudin 3 and Claudin 4

Total RNA was extracted from tissue biopsies using guanidium isothiocyanate (GITC, Catalog #: 50983, Sigma). The concentration and purity of extracted RNA was determined using a Nano Drop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) at 260 and 280 nm wavelength.

Complementary DNA (cDNA) synthesis

The extracted RNA was first converted into cDNA using reverse transcriptase reaction. In brief, one microgram of RNA from each grade of sample was used to convert cDNA using standard protocol of 10 min incubation with Oligo dT at 65°C using a thermal cycler followed by 2 min of snap cooling of the reaction. Further, a reaction mixture of 10 mM dNTPs, 1X reaction buffer with DTT and 1 unit of MMLV reverse transcriptase enzyme was added in the reaction mixture and incubated at 42°C for 45 min followed by 72°C for 10 min. The integrity of constructed cDNA was checked on 1 % agarose gel and 2 µL of cDNA was used for SYBR Green-based real-time quantitative polymerase chain reaction (RT-qPCR) to quantify CLDN-3 and CLDN-4 expression using respective cDNA.

RT-qPCR of Claudin 3 and Claudin 4 using SYBR-Green assay

RT-qPCR was performed using Real-time PCR machine (ABI 7500, Applied Biosystems, USA) with CLDN-3 (forward primer: 5′-ACCACCACCACCAACACC -3′, reverse primer: 5′-TGAGGTTTTACAGTCCATGC-3′) and CLDN-4 (forward primer: 5′-GCGTGCAGATAATGACAAGG′, reverse primer 5′-GGATTTGACGGCTCCTCTAC-3′); and GAPDH (forward primer: 5′-CAAGGTCATCCATGACAACTTTG-3′; reverser primer: 5′-GTCCACCACCCTGTTGCTGTAG-3′) primers. For each sample PCR reaction was performed in triplicate for Claudin 3, Claudin 4 and GAPDH separately, in a total of 20 µL reaction mixture. Following reaction conditions were set to determine cycle threshold (Ct) values for Claudin 3, Claudin 4 and GAPDH. Steps involved in PCR cycle were: (1) denaturation at 94 ◦ C for 2 min (2) denaturation at 94 ◦ C for 30s total of 40 cycles (3) annealing at 56 ◦ C for 30s (4) extension at 72°C for 30s. Further a single step of 10 min of melting curve was set to differentiate between primer-dimer and amplicons.

Statistical analysis

All the data are presented as mean ± standard deviation (SD). Student t-test was used to compare two groups and one way ANOVA was used to compare multiple groups. Statistical analysis was performed and data were presented using GraphPad Prism software (version 8.4.2). The statistical significance for all the groups was set as $p \leq 0.05$, $p \leq 0.005$, $p \leq 0.0001$ at 95 % CI.

RESULTS

Table 1: Demographic details and clinical characteristics of study participants with or without endometriosis

	Control (C), n=50, Mean (SD)	Patients with endometriosis, n=200; Each grade n=50, Mean (SD)				p value
		Grade 1 (G1)	Grade 2 (G2)	Grade 3 (G3)	Grade 4 (G4)	
Age, y	31.55 (2.77)	31.38 (2.44)	30.97 (2.56)	31.02 (2.71)	31.42 (2.58)	ns
Age of menarche, y	12.89 (1.35)	13.08 (1.29)	13.12 (1.32)	12.76 (1.23)	13.04 (1.28)	ns
Body mass index (kg/m^2)	22.54 (1.11)	22.48 (1.0)	22.52 (1.22)	22.50 (1.34)	22.42 (1.18)	ns
Duration of period pain, y	12.91 (1.33)	13.34 (1.06)	14.02 (1.20)	15.02 (1.56)	15.85 (1.95)	ns (C Vs. G1) <0.005 (C Vs. G2) <0.0001 (C Vs. G3) <0.0001 (C Vs. G4)
Menstrual pain, NRS	5.55 (1.31)	6.02(1.42)	6.53 (1.21)	7.02 (1.25)	7.63 (0.81)	ns (C Vs. G1) <0.005 (C Vs. G2) <0.0001 (C Vs. G3) <0.0001 (C Vs. G4)
Abdominal cramps	5.10 (1.0)	5.55 (0.92)	6.04 (1.17)	6.63 (1.24)	7.04 (0.85)	ns (C Vs. G1) <0.0005 (C Vs. G2) <0.0001 (C Vs. G3) <0.0001 (C Vs. G4)
Discomfort	5.21 (1.02)	5.72 (0.74)	6.29 (0.74)	6.82 (0.86)	7.34 (1.02)	ns (C Vs. G1) <0.0001 (C Vs. G2) <0.0001 (C Vs. G3) <0.0001 (C Vs. G4)

y, years; SD, standard deviation; NRS, numeric rating scale (0-10); ns, not significant

Demographic details and clinical characteristics of patients with different grades of endometriosis were presented in *table 1*. Significant differences in age, age of menarche and body mass index were not found when different grades of endometriosis were compared with control. However clinical characteristics such as duration of period pain, menstrual pain, abdominal cramps and discomfort progressively increased from grade 1 to grade 4 compared with control. In all the clinical characteristics, except the difference between control and grade 1, statistically significant differences were observed between control and grade 2, control and grade 3, and control and grade 4. Primary infertility and secondary infertility (% of total subjects) respectively in the groups were as follows: 2% (1 out of 50) and 4% (2 out of 50) in control; 10% (5 out of 50) and 12% (6 out of 50) in grade 1; 18% (9 out of 50) and 26% (13 out of 50) in grade 2; 24% (12 out of 50) and 28% (14 out of 50) in grade 3; 26% (13 out of 50) and 32% (16 out of 50) in grade 4. The trend in infertility, both primary and secondary, suggested an increase in percentage of patients from grade 1 to grade 4 when compared with control.

For microscopic examination of cells isolated from endometrial tissue biopsies, we used invitro culture model as a representative of invivo cells in different stages of endometriosis (*Figure* 1). Cells were

incubated in nutrient rich culture media and observed after 5 days of seeding. Studying the morphological characters of endometrial cells after day 5 demonstrated that cells in grade 1 and grade 2 exhibited epithelial phenotype and cells in grade 4 and grade 5 exhibited mesenchymal phenotype. Observing endometrial cell structure as we move from grade 1 to grade 4 illustrated gradual changeover from spherical shape (grade 1) to spindle/elongated shape (grade 4).

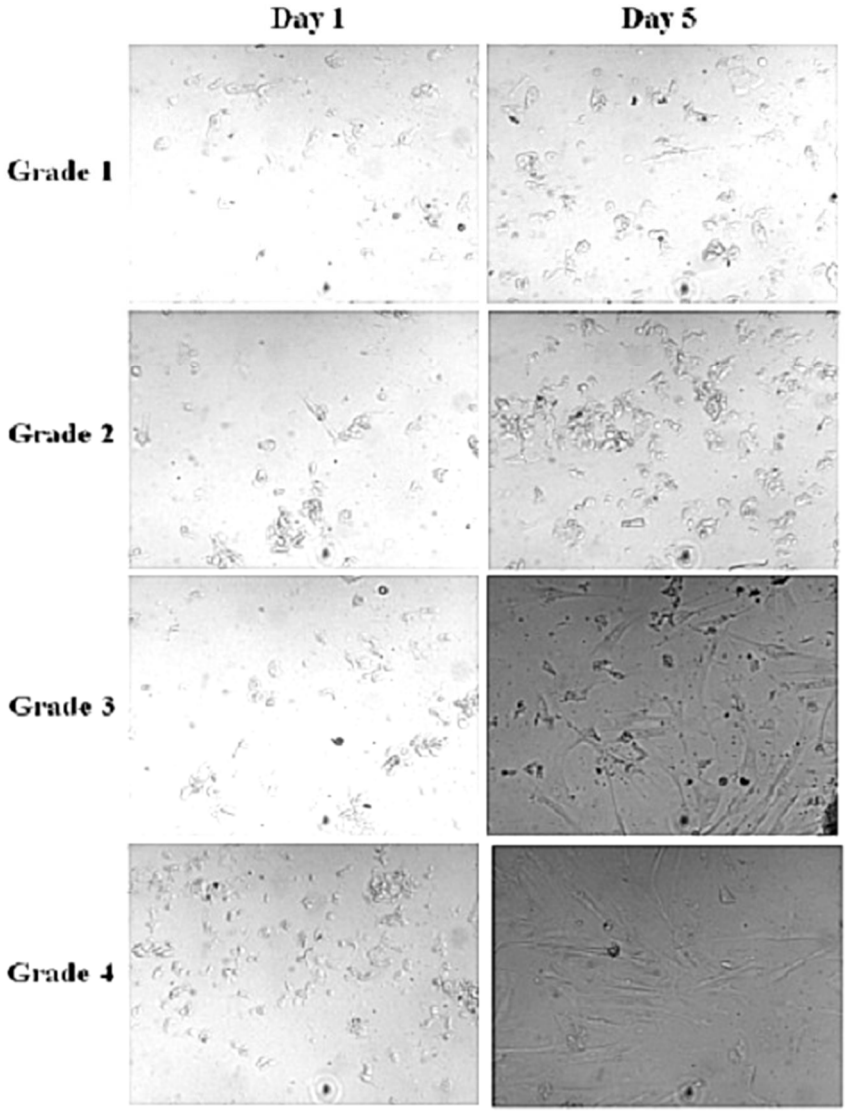


Figure 1: Microscopy observation showing morphology of cells derived from grade 1, 2, 3, and 4 endometrial tissues at day 1 of seeding and at day 5 (magnification: 10×, scale bar: 50 μm)

To add further, flow cytometry analysis of cells from different grades of endometriosis was performed using mesenchymal specific markers (CD-90, CD-73 and CD 105), which are absent in epithelial cells.[33] We observed more than 70% of transition from epithelial to mesenchymal cells in grade 3 and grade 4. These results showed an increase in percentage of cells expressing mesenchymal markers in grade 3 and grade 4 when compared with grade 1, grade 2 and control tissue biopsies (*Figure* 2).

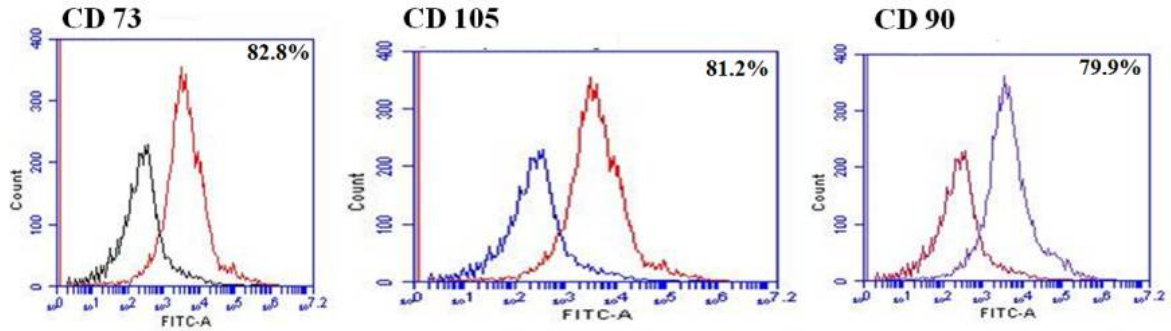


Figure 2: Flow-cytometry analysis of different grades of endometriosis using mesenchymal specific/stromal cell markers CD-73, CD-105, CD-90.

Study of claudin-3 expression profiles of different grades of endometriosis revealed a 25% decrease in the expression of claudin-3 in all the stages of endometriosis (combined together) when compared with the control group. When the claudin-3 expression was compared across different grades with the control group it showcased that in grade 3 and grade 4, claudin-3 expression is 60% and 40% of the control group respectively. Though slight variations in claudin-3 expression were evident in grade 1 and grade 2 when compared with the control group, the effects were not statistically significant (*Figure* 3).

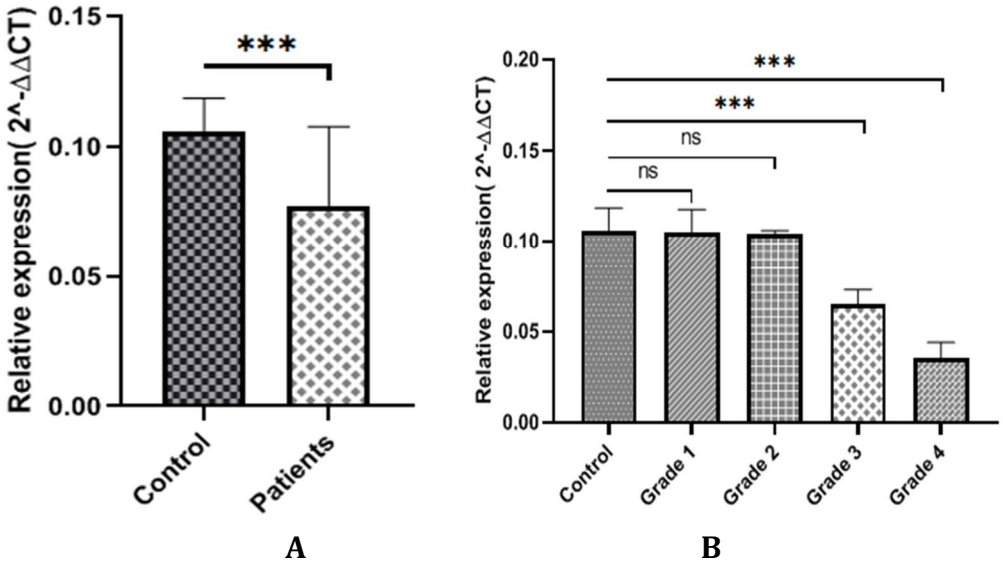
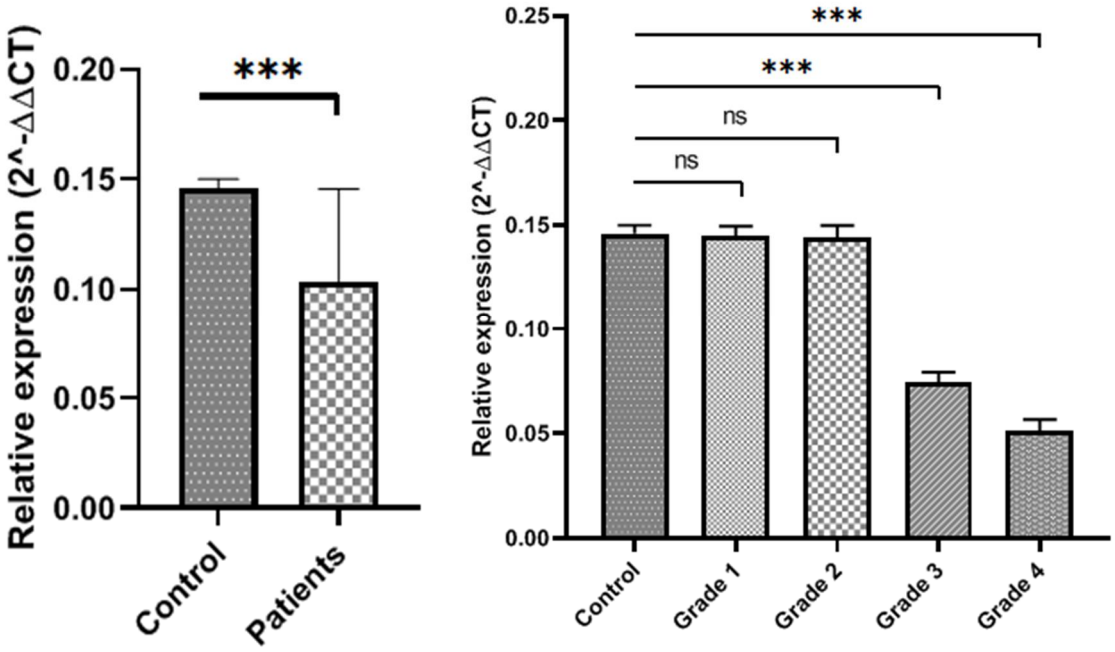


Figure 3: Differential expression of claudin-3 in endometriosis

(A) Relative fold change in claudin-3 expression in all the grades of endometriosis (combined together) compared with the control group. N = 50 for the control group and 200 for the patients group. Significant differences ($p<0.0001$, Mann-Whitney test) between the groups is indicated by a (***). Non significance is indicated by the abbreviation 'ns'. Values are mean +/- SD.

(B) Relative fold change in claudin-3 expression in different grades of endometriosis (grade 1, 2, 3 and 4) when compared with control. N = 50 for each group. Significant differences ($p<0.0001$, One way ANOVA) between the groups is indicated by a (***). Non significance is indicated by the abbreviation 'ns'. Values are mean +/- SD.

In a similar manner, examination of claudin-4 expression profiles of different grades of endometriosis revealed 24% decreased expression of claudin 4 in all the stages of endometriosis (combined together) when compared with the control group. When the claudin-4 expression was compared across different grades with the control group, it was manifested that in grade 3 and grade 4 claudin-3 expression was 65% and 35% of the control group respectively. Despite changes in claudin-4 expression in grade 1 and grade 2 when compared with the control group, the results lack statistical significance (*Figure* 4).



A **B**

Figure 4: Differential expression of claudin-4 in endometriosis

(A) Relative fold change in claudin-3 expression in all the grades of endometriosis (combined together) compared with the control group. N = 50 for the control group and 200 for the patients group. Significant differences ($p<0.0001$, Mann-Whitney test) between the groups is indicated by a (***). Non significance is indicated by the abbreviation 'ns'. Values are mean +/- SD.

(B) Relative fold change in claudin-3 expression in different grades of endometriosis (grade 1, 2, 3 and 4) when compared with control. N = 50 for each group. Significant differences ($p<0.0001$, One way ANOVA) between the groups is indicated by a (***). Non significance is indicated by the abbreviation 'ns'. Values are mean +/- SD.

DISCUSSION

Endometriosis, a pathological condition in women, is often classified into several grades based on the severity of the disease. Changes associated with different grades of endometriosis include alteration in cellular morphology and differences in gene expression of several proteins. Among the myriad of proteins that are altered in endometriosis, claudin (claudin 3 and 4 - gap junction proteins) role in pathogenesis has created considerable interest for several researchers. All these changes are an integral part of EMT, which is one of widely investigated theories that encompass step by step events.

EMT generally involves a series of steps: (a) Firstly, epithelial cell-cell contacts (tight junctions, adherens junctions, desmosomes and gap junctions, and hemi-desmosomes) are disrupted. (b) Secondly, cells lose their polarity through degradation of Crumbs, partitioning defective (PAR) and Scribble (SCRIB) polarity complexes. (c) Thirdly, expression of epithelial genes is turned off with concomitant activation of mesenchymal/stromal cell genes. (d) Finally, actin architecture is reorganized by forming lamellipodia, filopodia and invadopodia, which brings invasiveness and motility to the cells [34]. All these steps are intricately connected with observations or outcomes in our study.

This study aims to understand the variations displayed by different grades of endometriosis. Tracking these discrepancies from grade 1 to grade 4 suggests gradual increments in clinical characteristics (duration of period pain, menstrual pain, abdominal cramps, discomfort, and infertility) and the detrimental effects were more striking in grade 3 and grade 4. This signifies that as the disease progresses from grade 1 to grade 4, symptoms connected with endometriosis aggravates. This makes it imperative to understand this progressive transition in grades so that it offers us new findings in endometriosis and equips us with tools to counter the disease. Adding further, microscopic examination of in-vitro cultured cells isolated from different grades of endometriosis was also performed to witness differences in cell structure associated with progression of endometriosis. Endometrial cells showed morphological changes after 5 days of seeding and these changes were more striking in grade 3 and grade 4 when compared with grade 1 and

grade 2. In grade 3 and 4 altered morphology (cells are spindle) is due to formation of lamellipodia, filopodia and invadopodia, which is the outcome of reshaping of cellular skeletal structure (*Figure* 1). Acquiring these new features makes the epithelial cells move and invade distant locations (extra uterine tissues), leading to endometriosis.

As mentioned earlier one of the steps in EMT is switching off epithelial cell genes and switching on mesenchymal cell genes, especially markers CD-90, CD-73 and CD-105. To understand this, flow cytometry analysis of cells isolated from different grades of endometriosis was performed. Increase in percentage of cells with CD-90, CD-73 and CD-105 markers in grade 3 and grade 4 when compared with control signals that epithelial cells are transformed into mesenchymal cells. In grade 1 and grade 2 percentages of cells with CD-90, CD-73 and CD-105 markers was very few (*Figure* 2). Studies also reported decrease in the expression of epithelial cell markers and increased stromal/mesenchymal markers in both the ovarian and peritoneal endometriotic lesions [18,35]. As these mesenchymal cells/stromal cells possess stem cell characteristics, once these cells lodge in unusual sites they replicate and proliferate forming endometrial tissues.

Next comes the role of claudin-3 and claudin-4 in endometriosis. A study reported down-regulation of claudin-3 and claudin-4 in ectopic endometrium, suggesting pathogenic role of claudins in of endometriosis [36]. In contrast, Hoerscher A *et al* reported localization of claudin-2 and claudin-3 is identical in eutopic and ectopic endometrium, and concluded that claudin-2 and claudin-3 do not contribute to the pathogenesis of endometriosis [37]. However, these studies are handicapped with small sample size and to address this limitation we took large sample size (total of 200 subjects with different grades of endometriosis and 50 control subjects without endometriosis). Moreover till date studies on the role of claudin-3 and claudin-4 on the Indian population have not been evaluated. As we cannot turn blind eye towards genetic variability, we investigated the role of claudin-3 and claudin-4 in Indian women who are suffering from endometriosis by collecting their tissue biopsies. In our study the role of claudin-3 and claudin-4 in different grades of endometriosis was investigated by examining their expression profile (m-RNA) using RT-PCR. The expression of claudin-3 and claudin-4 in grades 3 and grade 4 was significantly lower when compared with control. On the other hand, changes in expression of claudin-3 and claudin-4 in grade 1 and grade 2 when compared with control were few and insignificant (*Figure* 3 and *figure* 4). Claudin, an integral component of tight junctions, is indispensable for cell-cell contact/integrity and epithelial cell homeostasis. Down regulation of claudin-3 and claudin-4 disrupts the tight junctions and liberates the cells from the basement membrane during the process of EMT transition, in fact this is the first step in EMT transition. The reduction or absence of claudin-3 and claudin-4 expression appears critical in the invasive phenotype of endometriotic cells. As cells are unshackled and begin to imbibe the mesenchymal cell phenotype, they pave the way for endometriosis. This signifies the importance of preservation of tight junctions (TJs) function to avert endometrial tissue pieces from attaching to the peritoneum.

In all the above three observations, ignoring the extent of statistical significance, we observed gradual transition in the characteristics (morphological changes, changes in percentage of cells expressing mesenchymal markers, and claudin-3 and claudin-4 expression changes) from grade 1 to grade 4 endometriosis when compared with control. Tracking these progressive changes from grade 1 to grade 4 endometriosis might assist us in forecasting the severity of disease, taking preventive measures, and developing biomarkers for prognosis.

Conclusion

Our study concludes that the findings in different grades of endometriosis and decreased expression of claudin-3 and claudin-4 in women with endometriosis might provide important clues in understanding the pathogenesis of endometriotic lesions in endometriosis.

Compliance with ethical standards

The present study is a prospective observational study with a written informed consent and was approved by institutional ethical committee.

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Competing interests

The authors have declared that no competing interest exists

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