Expression of Dkk 1 in Endometrial Endometrioid Carcinoma & Its Correlation with Wnt / β-catenin Signaling Pathway

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Abstract

Objective: Endometrial cancer is the most common form of cancer affecting female reproductive organs. Most common histologic type endometrioid carcinoma constitutes 75 to 80% of all cases. Studies on Dkk1 expression profiles and its inhibitory role in Wnt signaling pathway in genesis and development of endometrial carcinoma are very few. This study aims to investigate Dkk1 expression in endometrial carcinoma and its correlation with Wnt/β-catenin pathway. Methods: A total of 160 formalin fixed paraffin embedded samples including 50 cases each of endometrial atypical hyperplasia and endometrioid endometrial carcinoma along with 30 cases each of proliferative and secretory endometrium were included in this study. We investigated expression pattern of Dkk1, E-cadherin, β-catenin and c-myc in endometrial atypical hyperplasia and carcinoma as well as compared with that of proliferative and secretory endometrium. Immunohistochemistry and analysis were performed from July, 2018 to June, 2020. Results: We showed decreasing pattern of immunopositivity for Dkk1, E-cadherin and β-catenin from proliferative/secretory endometrium to endometrial atypical hyperplasia and endometrioid carcinoma. Increasing c-myc immunopositivity was noted from proliferative/secretory endometrium to endometrial atypical hyperplasia and endometrioid carcinoma. Moreover,
decreasing Dkk1 immunopositivity was well correlated with both E-cadherin, β-catenin and c-myc immunopositivity. **Conclusion:** Decreasing Dkk1 positivity from benign endometrium to endometrioid carcinoma suggests a negative regulatory function of Dkk1 in endometrioid carcinoma. Dkk1 is downregulated in Wnt signaling pathway in endometrioid endometrial carcinoma. Thus, Dkk1 can show promise as a biomarker for screening endometrioid carcinoma. Future researches can study the reactivation of the Dkk1 gene that could be a valuable strategy for antagonizing Wnt signaling pathway.

**Keywords:** Endometrioid carcinoma, Dkk1, Wnt/β-catenin pathway, β-catenin, E-cadherin

**Advances in Knowledge**
- Dkk1 shows decreasing trend of immunoexpression from benign phase endometrium to endometrioid endometrial carcinoma.
- Expression of Dkk1 is well correlated with the markers (β-catenin, E-cadherin, c-myc) of Wnt signaling pathway.
- Dkk1 has an antagonistic role in Wnt signaling pathway.

**Application to Patient Care**
- Dkk1 can be a promising biomarker in screening progression of endometrioid endometrial carcinoma.
- Reactivation of Dkk1 gene could be a valuable strategy to antagonize Wnt signaling pathway in endometrioid endometrial carcinoma.

**Introduction**
Endometrial cancer is the most prevalent invasive gynecologic malignancy among American women accounting for 7% of estimated new cancer cases in 2021. Incidence and death rates of endometrial cancer have been increasing by an average of 1.1% and 0.3% per year respectively. The most common histological type, endometrioid adenocarcinoma constitutes 75-80% of endometrial cancers. The disease mostly affects postmenopausal women with an average age of 60 years at diagnosis, while in women younger than 40 years it constitutes only five percent. In India it ranks third among female genital tract malignancies, after carcinoma cervix and carcinoma ovary. Most of the cases are diagnosed in early stages because of abnormal uterine
bleeding. The best diagnostic strategy in postmenopausal patient presenting with abnormal uterine bleeding, still remains controversial. Nowadays, endometrial biopsy and hysteroscopy have almost replaced dilatation and curettage (D&C) for the diagnosis and management of endometrioid carcinoma. Recent studies showed that the first step in the diagnostic pathway should be the measurement of endometrial thickness, followed by endometrial sampling. Clinical assessment, radiological evaluation and histopathological examination have led the way to study of molecular pathways like Wnt signaling pathway. Wnt signal transduction pathway is activated by binding of a Wnt protein to cell surface receptor. E-cadherin (a cell adhesion molecule forming adherens junctions between cells), β-catenin (a subunit of cadherin protein complex) and c-myc (a transcription factor protein regulating cell proliferation) are integral components of Wnt signaling pathway. Abnormalities of Wnt signaling transduction pathway [Figure 1] is responsible for genesis and development of some human malignant tumors. Attempts have been made to investigate various regulators in the Wnt signaling pathway as targets for diagnosis and treatment of malignant tumors. Several candidate markers, such as E-cadherin, β-catenin, c-myc and others have been proposed for use on cytologic or histologic samples in endometrial carcinoma. As a negative regulator in Wnt signaling pathway, Dkk1 can inhibit Wnt activation in tumor progression. Earlier studies in colorectum and placenta showed that Dkk1 was prominently expressed in normal cells but absent in cancer cells. At present, studies on Dkk1 expression profiles in endometrial carcinoma are very few. Dkk1 expression pattern in endometrial carcinoma and its correlation with other components of Wnt pathway, especially β-catenin, E-cadherin and c-myc has not been studied so far in India. This study will investigate the expression pattern of Dkk1, E-cadherin, β-catenin and c-myc in endometrial carcinoma. Moreover, the expression pattern of these markers in endometrial atypical hyperplasia and carcinoma will be compared with that of proliferative and secretory endometrium.

Methods

Selection of Cases

This retrospective study was conducted at the Department of Pathology where formalin fixed paraffin embedded (FFPE) samples of endometrial lesions, age ranging from 21 to 77 years, collected between January 2005 and March 2018, were selected including 50 cases each of
endometrial atypical hyperplasia and endometrioid endometrial carcinoma along with 30 cases of proliferative and secretory endometrium. Endometrial samples in younger patients were taken primarily to exclude the causes of infertility and abnormal uterine bleeding. Standard morphological criteria were used for diagnosis and selection of cases and control groups. The study was approved by the Institutional Ethics Committee. One section of each sample was stained with hematoxylin and eosin (H&E) and four step sections on coated slides were used for Dkk1, E-cadherin, β-catenin and c-myc immunohistochemistry (IHC). Immunohistochemistry and analysis were performed over the next 2 years from July, 2018 to June, 2020.

**Immunohistochemistry**

Immunohistochemistry was performed using available monoclonal antibodies for Dkk1, E-cadherin, β-catenin and c-myc (Dkk1, Abcam, 1:100; β-catenin, Thermo Scientific, 1:400; c-myc, Thermo Scientific, 1:100; and E-cadherin, Thermo Scientific, 1:200).

**Steps.** Serial 4-micron thick sections were cut from the selected representative paraffin embedded tissue blocks and 3-aminopropyl triethoxysilane (APTES) coated slides were used for IHC. Slides were deparaffinized, followed by rehydration in decreasing concentration of alcohol. For Dkk1, E-cadherin and c-myc immunostains, antigen retrieval was done by heating the sections in citrate buffer inside a 600 watt microwave oven at full power for 30 minutes. For β-catenin Tris-EDTA buffer at pH 8 was used for heat mediated antigen retrieval. To diminish the nonspecific immunostaining (i.e. endogenous peroxidase activity), each slide was treated with methanol containing 4% hydrogen peroxide for 30 minutes. For all immunostains, sections were then overlaid with adequate amount of appropriately diluted primary antibody followed by overnight incubation at 4°C in a humid chamber. After 3 changes of washing (5 minutes each) in Tris- HCl buffer peroxidase conjugated streptavidin was applied to cover the sections and incubated at room temperature for 30 minutes. Each section was then covered with substrate chromogen solution freshly prepared by dissolving 50 µl of Di-amino Benzidine (DAB) chromogen to 1 ml of DAB substrate buffer. The sections were counterstained with hematoxylin for 10 seconds, followed by mounting with DPX. During staining of each batch, appropriate positive and negative controls (by omitting primary antibody) were used.
Analysis. IHC stains (Dkk1, cytoplasmic; β-catenin, membranous; c-myc, cytoplasmic and nuclear; E-caderin, membranous) were reviewed and analysed in conjunction with hematoxylin and eosin (H&E) stained slides. Immunoreactive score (IRS) was obtained by multiplying intensity score (0, no staining; 1, weak; 2, moderate and 3 strong staining) and percentage score (0, nil; 1, <10%; 2, 10-50%; 3, 51-80% and 4, >80%). Thus, the total IRS score ranged from 0 to 12.13 Two independent observers had analyzed the expression pattern of all four markers and then an average was calculated for final analysis. Appropriate statistical tests including independent sample t test, Chi-square test and Pearson correlation test were applied to analyze the significance of results between cases and control groups using the Statistical Package for the Social Sciences (SPSS), version 21.0 (IBM Inc., Chicago, Illinois, USA) software program. The P<0.05 was considered statistically significant.

Results

The retrospective study evaluated a total number of 160 samples including proliferative endometrium, secretory endometrium, atypical hyperplasia and endometrioid carcinoma. Immunoprofiles using Dkk1, E-cadherin, c-myc and β-catenin were compiled, compared and analyzed for different expression pattern in various groups of endometrium.

Age Distribution. Age pattern of proliferative group versus secretory group was statistically insignificant (P value 1.000), while the age patterns between proliferative endometrium versus endometrial atypical hyperplasia; proliferative endometrium versus endometrial carcinoma; secretory endometrium versus endometrial atypical hyperplasia; secretory endometrium versus endometrial carcinoma; as well as endometrial atypical hyperplasia versus endometrial carcinoma were statistically significant (P value <0.001).

Intergroup Dkk1 Immunopositivity. Dkk1 showed mostly cytoplasmic expression in glandular epithelium during proliferative phase, endometrial atypical hyperplasia and endometrioid carcinoma. However, 2 cases of proliferative endometrium had nonspecific nuclear positivity both in glandular epithelium and the stroma. Secretory endometrium showed cytoplasmic immunopositivity both in glandular as well as stromal cells. Squamous morules associated with endometrioid carcinoma also had similar cytoplasmic immunopositivity. We have studied
cytoplasmic expression among the groups. Dkk1 immunopositivity of proliferative endometrium versus secretory endometrium was statistically insignificant ($P$ value 0.183). There was increased Dkk1 immunopositivity in proliferative endometrium as compared to endometrial atypical hyperplasia and endometrioid carcinoma [Figure 2], which was statistically significant ($P$ value <0.001). Dkk1 immunopositivity of endometrial atypical hyperplasia versus endometrioid carcinoma was statistically insignificant ($P$ value 1.000). Secretory endometrium showed increased Dkk1 immunopositivity as compared to endometrial atypical hyperplasia and endometrioid carcinoma and the difference was statistically significant ($P$ value <0.001). Dkk1 showed decreasing trend of expression from endometrial atypical hyperplasia to grade I endometrioid carcinoma to grade II endometrioid carcinoma. When individual grades are compared separately, the difference between endometrial atypical hyperplasia and grade I endometrioid carcinoma was statistically insignificant ($P$ value 1.000), but it was statistically significant in between endometrial atypical hyperplasia and grade II endometrioid carcinoma ($P$ value 0.048).

**Intergroup E-cadherin Immunopositivity.** E-cadherin showed membranous immunopositivity. E-cadherin immunopositivity of proliferative endometrium versus secretory endometrium was statistically insignificant ($P$ value 1.000). Immunopositivity of both proliferative endometrium and secretory endometrium were higher than that of endometrial atypical hyperplasia and endometrioid carcinoma [Figure 3]; and the difference in immunopositivity among them were statistically significant ($P$ value <0.001). There was also statistically significant difference between endometrial atypical hyperplasia and endometrioid carcinoma ($P$ value <0.001).

**Intergroup β-catenin Immunopositivity.** Membranous β-catenin expression was studied among the groups. Nuclear β-catenin was observed in 14% (7/50) of endometrioid carcinoma excluding the areas of squamous morule formation that also showed nuclear positivity. β-catenin immunopositivity of proliferative endometrium versus secretory endometrium was statistically insignificant ($P$ value 1.000). In this study, both proliferative endometrium and secretory endometrium showed increased immunopositivity of β-catenin as compared to endometrial atypical hyperplasia and endometrioid carcinoma [Figure 4]; and the difference in β-catenin immunopositivity among them were statistically significant ($P$ value <0.001). Immunopositivity
in endometrial atypical hyperplasia was statistically significant ($P$ value <0.001) when compared to that of endometrial carcinoma.

**Intergroup c-myc Immunopositivity.** We evaluated cytoplasmic c-myc immunopositivity among the groups. Additionally, nuclear expression was noted in 14 cases and 4 cases of proliferative and secretory endometrium respectively. When c-myc immunopositivity of proliferative endometrium versus secretory endometrium was compared, the difference was statistically insignificant ($P$ value 1.000). There was increased immunopositivity in endometrioid carcinoma as compared to proliferative endometrium and endometrial atypical hyperplasia [Figure 5]; the difference in c-myc immunopositivity among them were statistically significant ($P$ value 0.043 and <0.001 respectively). By contrast, c-myc immunopositivity of secretory endometrium versus endometrial atypical hyperplasia was statistically insignificant ($P$ value 0.384), while c-myc immunopositivity of secretory endometrium versus endometrioid carcinoma was statistically significant ($P$ value <0.001).

**Intragroup Correlation among Immunohistochemistry Markers.** In endometrial atypical hyperplasia group, we found statistically significant correlation between Dkk1 and β-catenin immunopositivity, as well as between E-cadherin and c-myc immunopositivity. Rest three groups didn’t show any significant correlation among the four IHC markers. Comparison of immunohistchemistry between two age groups in endometrial atypical hyperplasia and endometrioid carcinoma as well as between grade I and grade II endometrioid carcinomas didn’t reveal any significant difference [Table 1].

**Discussion**

Endometrial cancer has surpassed cervical cancer as the most common gynecologic malignancy. Cervical cancer was much more prevalent in past few decades compared to endometrial cancer, but earlier detection and eradication of cervical precursor lesions has reversed the ratio.

Endometrial carcinoma frequently occurs in peri-and post-menopausal women with endometrioid carcinoma being the most common histological subtype. PTEN genetic mutation is most frequent (39-83%) in endometrioid cancer, however β-catenin mutation accounts for 31-47% of the cases. β-catenin is an integral component of Wnt signaling
pathway [Figure 1], that is dysregulated in many human cancers. On contrary, a negative regulator of β-catenin pathway, Dkk1 prevents tumor progression by inhibiting this signaling pathway.9 Some studies described role of Dkk1 in non-endometrial tissues both in normal and corresponding malignant cells, however studies on endometrial cancer are very less in English literature.11,12 Hence, we have tried to evaluate expression pattern of Dkk1 in various groups of benign, atypical and malignant endometrium as well as correlated with other markers like E-cadherin, β-catenin, c-myc of Wnt pathway to show their relation among the groups.

Dkk1. Dkk1 is a glycoprotein and one of the members of Dkk family (Dkks), secreted by various cells throughout the human body.17 The human Dkk1 gene maps to chromosome 10q11.2, which encodes a protein that acts as an antagonist in Wnt signaling pathway [Figure 1C] by binding to and inhibiting LRP 5/6.18 Yi N et al showed Dkk1 positivity both in benign endometrium and endometrial carcinoma, where Dkk1 was mostly distributed in the cytoplasm of glandular epithelium. They have documented ‘high expression’ of Dkk1 predominantly in benign endometrium, in contrast to “low expression” in endometrial cancer suggesting that this reduction expression may be due to its negative regulatory function in Wnt signaling pathway.12 We also found decreasing Dkk1 immunopositivity from proliferative/secretory endometrium to endometrial atypical hyperplasia and endometrioid carcinoma. In our study Dkk1 positivity was predominantly in the cytoplasm of glandular epithelium, however stromal cells also showed weak cytoplasmic immunopositivity [Figure 2]. We also found significant difference in Dkk1 immunopositivity between endometrial atypical hyperplasia and proliferative/secretory endometrium; as well as between endometrioid carcinoma and proliferative/secretory endometrium. Though there was increased Dkk1 immunopositivity in endometrial atypical hyperplasia as compared to endometrioid carcinoma, it did not achieve statistical significance. Interestingly some studies demonstrated reduced expression of β-catenin following treatment with exogenous Dkk1 probably indicating that increased Dkk1 binding to LRP5/6 inhibits Wnt signaling leading to degradation of β-catenin.19 Decreasing Dkk1 positivity in our study from benign endometrium to endometrioid carcinoma may suggest that negative regulatory function of Dkk1 is reduced from benign to malignant endometrium. Thus at least in part, by inducing abnormalities of Wnt signaling pathway, Dkk1 plays a role in the genesis and development of endometrial carcinoma. Similar patterns of Dkk1 alterations have also been reported in some
other tumors including colorectal cancer, placental choriocarcinoma and non-small cell lung cancers where Dkk genes were frequently silenced. In our study decreasing positivity of Dkk1 from proliferative/secretory endometrium to endometrial atypical hyperplasia and endometrioid carcinoma, suggests that Dkk1 is involved in the early phase of endometrioid carcinoma by suppressing Wnt pathway.

E-cadherin. Cell surface glycoprotein E-cadherin with a molecular weight of 120 kDa is a major cadherin molecule expressed by epithelial cells. It binds to catenin [Figure 1A] to form a cadherin-catenin complex that plays an important role in intercellular adhesion. Shih et al demonstrated that the cytoplasmic expression of E-cadherin in endometrial glandular cells occurred mainly in the proliferative phase and decreased in the secretory phase. In contrast to this study we found strong membranous immunopositivity both in proliferative and secretory endometrium. Although, similar to their study, we found decreased E-cadherin expression in endometrioid carcinoma as compared to proliferative/secretory endometrium. The mechanism of reduced of E-cadherin positivity has not been fully understood, however, Saito et al showed that loss of E-cadherin positivity was caused by promoter methylation of the E-cadherin gene. In our study, we found significant difference in E-cadherin immunopositivity between endometrial atypical hyperplasia and proliferative/secretory endometrium; as well as between endometrioid carcinoma and proliferative/secretory endometrium. We also showed that E-cadherin immunopositivity was significantly different between endometrial atypical hyperplasia and endometrioid carcinoma. So far, none of the previous studies has mentioned difference in E-cadherin positivity between endometrial atypical hyperplasia and carcinoma.

β-catenin. β-catenin encoded by CTNNB1 gene is a subunit of the cadherin protein complex. It takes part in the formation of adherens junctions [Figure 1], that plays a pivotal role in maintaining epithelial cell layers by regulating cellular adhesion and growth signals. Several studies showed that it has been implicated in the pathogenesis and progression of many human malignancies involving Wnt pathway. As a signal transducer in Wnt pathway it induces targeted gene expression and cytoplasmic β-catenin accumulation. Previous studies demonstrated greater positivity of cytoplasmic β-catenin in the glandular cells of proliferative endometrium as compared to secretory phase. These studies also showed nuclear positivity of β-catenin in the
glandular cells of the proliferative and early secretory phase endometrium. However, we did not find any difference in β-catenin immunopositivity between proliferative and secretory endometrium as well as no nuclear β-catenin immunoactivity in proliferative/secretory endometrium or in endometrial atypical hyperplasia. Shih et al revealed that the nuclear β-catenin-positive cells lacked E-cadherin positivity which indicated an inverse correlation between E-cadherin and nuclear β-catenin positivity. This result was concordant with our study where 14% of endometrioid carcinoma showed nuclear β-catenin immunopositivity, and most of them showed near total loss of membranous E-cadherin immunopositivity. Exact mechanisms behind this reduced positivity of E-cadherin at nuclear β-catenin positive sites are still not elucidated, however it may be due to nuclear translocation of β-catenin that impairs the β-catenin/E-cadherin adherent junction complex that finally leads to E-cadherin release from the cell membrane.

The mechanisms of nuclear accumulation of β-catenin are reported to be responsible for the mutation of β-catenin and related genes. Studies on Wnt pathway in colorectal cancers demonstrated β-catenin stabilization and its significant accumulation in the cell which were primarily attributed to the mutation of the adenomatosis polyposis coli (APC) or β-catenin gene in the signaling pathway resulting in cell cycle progression in colorectal cancer. Our study showed decreasing membranous immunopositivity of β-catenin from proliferative/secretory endometrium to endometrial atypical hyperplasia and endometrioid carcinoma. We also showed that there was significant difference in β-catenin immunopositivity between endometrial atypical hyperplasia and proliferative/secretory endometrium; between endometrioid carcinoma and proliferative/secretory endometrium; as well as between endometrial atypical hyperplasia and endometrioid carcinoma. In this study, nuclear β-catenin positive cases of endometrioid carcinoma showed increased cytoplasmic c-myc immunopositivity. Hence, both c-myc and β-catenin were found to be upregulated in these cases of endometrioid carcinomas.

c-myc. c-myc is a nuclear DNA binding protein that is implicated in cell cycle regulation. c-myc amplifications in many human cancers were found to be associated with tumor aggressiveness and poor prognosis. A cyclic variation in the c-myc positivity was reported by Odom et al with higher expression in the proliferative than in the secretory phase. In contrast to this finding, we
observed increased c-myc immunopositivity in secretory endometrium as compared to proliferative endometrium. Bircan et al in their study showed that the anti c-myc monoclonal antibody was detected both in the nucleus and the cytoplasm, which was concordant with our study. Actively dividing cells of proliferative phase endometrium displayed a nuclear distribution, while in differentiated cells of the secretory phase the immunostaining was primarily cytoplasmic. They showed cytoplasmic and perinuclear c-myc positivity in 15.3% of endometrial cancers. Another study by Geisler et al demonstrated both cytoplasmic and nuclear c-myc immunopositivity in 75.2% and 66.9% of cases of endometrial cancers respectively. By contrast, we found only cytoplasmic c-myc immunopositivity in all cases of endometrioid carcinomas along with few cases of proliferative and secretory endometrium showing nuclear c-myc immunopositivity. We also found increasing cytoplasmic immunopositivity of c-myc from proliferative/secretory endometrium to endometrial atypical hyperplasia and endometrioid carcinoma. There was also significant difference in immunopositivity between endometrial atypical hyperplasia and proliferative endometrium; between endometrioid carcinoma and proliferative/secretory endometrium; as well as between endometrial atypical hyperplasia and carcinoma. However, we did not find any significant difference in c-myc immunopositivity between endometrial atypical hyperplasia and secretory endometrium.

Conclusion
Decreasing Dkk1 immunopositivity from proliferative/secretory endometrium to endometrial atypical hyperplasia to endometrioid carcinoma indicates that Dkk1 is downregulated in endometrioid endometrial carcinoma. Immunoprofiles of Dkk1 and the other markers associated with Wnt signaling pathway explain the antagonistic role of Dkk1 in the Wnt signaling pathway in endometrial cancer. Thus, Dkk1 shows promise as a biomarker for screening progression of endometrioid carcinoma. On the other hand, reactivation of the Dkk1 gene could be a valuable strategy for antagonizing Wnt signaling pathway.

Conflicts of Interest
The authors declare no conflict of interests.
Funding
No funding was received for this study.

Author Contributions
AD and SM conceptualised and designed the study. SM drafted the manuscript. SK and NB performed critical review and contributed with suggestions. SM and AD were involved in data collection, data entry, literature search, and data analysis. All the authors approved the final version of the manuscript.

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Figure 1: A) Absence of signaling molecule i.e., legend (Wnt molecule) leads to formation of ‘destruction complex’ that in turn creates a hyperphosphorylated β-catenin destined for proteosomal degradation. Also depicted is E-cadherin binding to β-catenin forming adherens junction. B) Wnt molecule binding to Frizzled (FZD)/LRP 5/6 receptors inactivates ‘destruction complex’ and stabilizes hypophosphorylated β-catenin that enter nucleus to interact with TCF/LEF family proteins to activate gene transcription. C) Dkk1 binds to LRP5/6 co-receptor and blocks Wnt binding that ultimately results in β-catenin degradation and repression of gene transcription. (Illustration is created by the authors).

Figure 2: Dkk1 immunopositivity. Proliferative endometrium (A, 400X magnification), secretory endometrium (B, 400X magnification), endometrial atypical hyperplasia (C, 400X magnification), and endometrioid carcinoma (D, 400X magnification).
**Figure 3:** E-cadherin immunopositivity. Proliferative endometrium (A, 400X magnification), secretory endometrium (B, 400X magnification), endometrial atypical hyperplasia (C, 400X magnification), and endometrioid carcinoma (D, 400X magnification).

**Figure 4:** β-catenin immunopositivity. Secretory endometrium (A, 400X magnification), endometrial atypical hyperplasia (B, 100X magnification), endometrioid carcinoma (C, 400X magnification), and nuclear positivity in endometrioid carcinoma (D, 400X magnification).

**Figure 5:** c-myc immunopositivity. Proliferative endometrium (A, 100X magnification), secretory endometrium (B, 200X magnification), endometrial atypical hyperplasia (C, 200X magnification), and endometrioid carcinoma (D, 400X magnification).
**Table 1:** Comparison of immunopositivity between grade I and grade II endometrioid carcinoma

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