Original Article

Prognostic significance of Notch signaling molecules and their involvement in the invasiveness of endometrial carcinoma cells

Yuko Mitsuhashi,[a] Akiko Horiuchi, [a] Tsutomu Miyamoto,[a] Hiroyasu Kashima,[a] Akihisa Suzuki,[a] and Tanri Shiozawa.[a]

a: Department of Obstetrics and Gynecology, Shinshu University School of Medicine, Japan

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Address correspondence and reprints request to: Akihisa Suzuki, M.D.

Department of Obstetrics and Gynecology, Shinshu University School of Medicine,

3-1-1 Asahi, Matsumoto 390-8621, Japan

Tel: 81-263-37-2718 Fax: 81-263-34-0944 e-mail: suzuaki@shinshu-u.ac.jp

Abstract

Aim: The aim was to investigate the significance of the expression of Notch-related molecules in endometrial carcinoma.

Methods and Results: The expression of Notch receptors (Notch 1 and 3) and Notch ligands (Jagged 1 and Delta-like 4) was examined immunohistochemically in 37 normal and 76 malignant endometrial tissue samples. The result of each staining was described as a positivity index (PI, full score; 200). The effects of a Notch inhibitor, DAPT, on cell proliferation, invasion, and motility were investigated using endometrial carcinoma cell lines. The PI for Notch 1 (90.4±15.3, mean±SD), Notch 3 (95.6±20.4), Jagged 1 (95.5±10.0), and Delta-like 4 (88.2±9.6), was significantly higher in endometrial carcinoma than normal endometrium. The PI for Notch 1 was significantly associated with advanced FIGO stage. In addition, patients with high expression of Notch1 and Jag1 carcinomas had a poor prognosis compared with those with double-negative carcinomas (P = .015). DAPT suppressed invasiveness of cells derived from the endometrial carcinoma cell line KLE.

Conclusions: Notch 1-Jagged 1 axis may enhance the invasive property of endometrial carcinomas, which suggest the Notch pathway to be a promising target in the treatment of this malignancy.

Introduction

Endometrial carcinoma is one of the most common malignancies in the female genital tract, [1] with an estimated 42160 cases of and 7780 deaths from the disease in the United States in 2009.[2] The number of patients with endometrial carcinoma of an advanced stage, indicative of a poor prognosis, is increasing along with the total number of cases.[3] Thus, understanding the invasive characteristics of endometrial carcinoma is important for better management of this disease.

Notch signaling participates in tissue development by maintaining the self-renewal potential of some tissues and can also influence proliferation, differentiation and apoptosis in various cell types.[4,5] Four Notch receptors have been identified (Notch 1 to 4) in humans along with five corresponding ligands, including Delta-like(DLL) 1, DLL3 and DLL4, and Jagged(JAG) 1 and JAG2.[6] After the activation of Notch receptors by ligand binding, Notch proteins are proteolytically cleaved in two steps by ADAM10 and γ-secretase, and the intracellular domain of Notch (NICD) is translocated to the nucleus. Then NICD interacts with the transcription factor CSL to regulate transcription of the basic helix-loop-helix protein hairy/enhancer of split (HES) and hairy/enhancer of split related protein (HERP).[7,8]

Recent studies have revealed that the activation of Notch signaling is involved in the development of T-cell acute lymphoblastic leukemia in various animal models[9] and the overexpression of Notch receptors with their ligands has been detected in many human solid cancers.[10-12] However, the expression and functional involvement of Notch signaling molecules in endometrial tissues remain undetermined. In the present study, we examined the immunohistochemical expression of representative Notch receptors (Notch1 and Notch3) and Notch ligands (JAG1 and DLL4) in normal and neoplastic endometrial tissues. In addition, the effects of Notch signaling on the proliferation and the invasiveness of endometrial carcinoma cells were examined using a γ-secretase

inhibitor, DAPT.

Materials and Methods

CELL LINES AND REAGENTS

The endometrial carcinoma cell lines Ishikawa, and Hec-1A and Hec1-B were kindly provided by Dr. H. Nishida at Kasumigaura Medical Center (Tsuchiura, Japan) and Dr. Kuramoto at Kitazato University (Sagamihara, Japan), respectively. HHUA was purchased from the Riken Cell Bank (Saitama, Japan) with the permission of Dr. Ishiwata at the Ishiwata Labaratory (Mito, Japan). KLE was purchased from American Type Culture Collection (Rockville, MD). These cells were cultured in recommended media (Table 1) and 1% antibiotics. ECL was purchased from Amersham (Piscataway, NJ). The Histofine SAB-PO detector kit was purchased from Nichirei (Tokyo, Japan). WST-1 reagent was obtained from Roche (Indianapolis, IN). DAPT was purchased from Calbiochem (San Diego, CA). Antibodies used in this study are listed in Table 2.

IMMUNOHISTOCHEMSTRY

Histological materials

Thirty-seven specimens of normal endometrium, obtained from women who underwent a hysterectomy for myoma uteri, were selected from the pathology files of Shinshu University Hospital. Nineteen of the specimens were in the proliferative phase and 18 were in the secretory phase. Seventy-six patients with endometrial endometrioid carcinoma visited Shinshu University Hospital between 1996 and 2005 and underwent a hysterectomy, bilateral salpingo-oophorectomy, and lymph node dissection or biopsy. The age of the patients ranged from 33 to 90 years (median 57). The clinical follow-up period ranged from 6 to 144 months (median, 59). According to the International Federation of Gynecology and Obstetrics (FIGO) classification (2009), 57 of the 76

patients were in stage I, 0 were in stage II, 11 were in stage III, and 8 were stage IV. With regard to histological grade, 50 tumors were of grade 1, 9 were of grade 2, and 17 were of grade 3.These specimens were fixed in 10% phosphate-buffered formalin and embedded in paraffin. Each tissue sample was used with the approval of the Ethics Committee of Shinshu University, after obtaining written consent from the patients. Serial 3-μ-thick sections were prepared for hematoxylin and eosin staining and immunostaining.

Staining procedures

Indirect immunohistochemical staining was performed by the avidin-biotin- peroxidase complex method using a Histofine SAB-PO detector kit as described previously.[13] Briefly, after deparaffinization in xylene and rehydration through graded concentrations of alcohol, each section was treated by microwave in 0.01 M citrate buffer (pH 6.0) for 15 min. Endogenous peroxidase activity was blocked by 0.03% hydrogen peroxide in methyl alcohol for 30 min. Then, 10% normal rabbit serum (for mouse primary antibodies), or goat serum (for rabbit primary antibodies), was applied to minimize non-specific reactivity. The dilution ratio of primary antibodies was shown in Table 2. The sections were then incubated with specific primary antibodies at 4°C overnight. After a rinse with PBS, biotinylated anti-mouse or anti-rabbit immunoglobulin (IgG) was applied for 30 min at room temperature. After a rinse with PBS, a peroxidase-conjugated streptavidin solution was applied for 30 min and the antigen-antibody reaction was visualized by 0.05% 3,3'-diaminobenzidine (DAB). The tissue was counterstained with hematoxylin.

Interpretation of immunohistochemical staining and statistical analysis

Immunoreactivity was evaluated by counting the number of positive cells among 200

arbitrarily selected cells in 3 high-power fields in each section. For the assessment of cytoplasmic staining, we separately evaluated the percentage of positive cells and staining intensity (negative, 0; weak, 1; strong, 2) under standard light microscopy. A positivity index (PI) was calculated by multiplying the percentage of positive cells (0-100) by the staining intensity (0-2) with a full score of 200. In the survival analysis, cut-off PI values for the staining of each molecule were determined in reference to the mean PI values of each staining. The significance of differences in the PI among histological grades was examined with the Mann Whitney U-test. A tied P-value of < 0.05 was considered significant. The association with clinicopathological parameters was analyzed by the chi-square test. To assess independent relationship between Notch-related molecules and disease stage (an outcome variable), a multivariate regression analysis was performed, initially including PIs for Notch-related molecules(Notch1, Notch2, JAG1, and DLL4), myometrial invasion (<1/2 vs. >1/2), vessel involvement, lymph node metastasis, cytology in ascites, ovarian metastasis, and cervical invasion. A backward stepwise elimination with a threshold of P=.10 was used to select variables in the final model. Cumulative survival was also analyzed using the Kaplan-Meier method. These analyses were conducted using the StatView system (Abacus, Berkeley, CA).

WESTERN BLOTTING

Proteins extracted from cells of sub-confluent cultures were subjected to a Western blot analysis as described previously.[14] Each tissue sample was used with written consent from the patient. Blocking was performed with 5% nonfat milk or 3% bovine serum albumin in PBS-T for 1 hour at room temperature. The membranes were blotted with primary antibody at 4^{o}C overnight and then incubated with a peroxidase-conjugated secondary antibody. Bound antibodies were visualized using the ECL Western blot

detection reagent. β-actin was used as a loading control.

PROLIFERATION ASSAY

Cell proliferation was examined using WST-1 proliferation reagent as described previously.[15]Cells were seeded into a 96-well multiplate (3 x 10^3 cells per well). After the cells became 50% confluent, DAPT was added at serial concentrations.

IN VITRO INVASION ASSAY

Cell invasion through a reconstituted basement membrane (Matrigel; BD Biosciences, Bedford, MA), was assayed by a method reported previously.[16] KLE cell was used for this analysis because it was representative of a poorly undifferentiated endometrial carcinoma and used for this analysis previously. [17] After 22 hours of incubation with DAPT or culture medium for KLE as a control, the invading cells were counted in five different fields under a light microscope at ×200 magnification and averaged.

MIGRATION ASSAY

Cell migration was examined using a monolayer wounding system.[18] In brief, KLE cells were seeded into 60mm dishes and incubated until confluent. Next, scratch lines were introduced using a plastic tip. Then, DAPT ($1x10^{-6}$M) or vehicle was added. The rate of motility of cells was calculated as the number of cells entering the central denuded area. Cell numbers were expressed as cells per mm^2 of the original denuded area.

CELL ADHESION ASSAY

KLE cells were treated with DAPT at $1x10^{-6}$M or vehicle for 24 hours. The cells were seeded at $5x10^4$ cells /well into 96-well plates coated with type IV collagen. After 2 hours of culture, the medium and non-adherent cells were removed and cells were

washed with culture medium. Adherent cells were stained with WST-1 reagent. After incubation for 3 hours, absorbance was measured.

Results

EXPRESSION OF NOTCH LIGANDS AND NOTCH RECEPTORS IN NORMAL ENDOMETRIUM

The PI values for Notch receptors (Notch1 and Notch3) and Notch ligands (DLL4 and JAG1) are summarized in Tables 3 and 4. In normal proliferative endometria, cytoplasmic staining for Notch1 and Notch3 in the glandular cells was diffuse and the mean PI values ± standard deviation (SD) for Notch1 and Notch3 in the functional layer were 72.1 ± 10.2 and 75.0 ± 6.0, respectively (Table 3; Figs. 1A and 1D). Similarly, the staining of Notch1 and Notch3 was observed in the secretory phase (Table 3; Figs. 1B and 1E). There was no significant difference in the PI values for Notch1 and Notch3 between the proliferative and secretory phases (Figs. 1C and 1F). The PI for Notch 1 and Notch 3 of the basal layer showed no significant difference compared with the functional layer (Figs.1C and 1F). The staining of Notch receptors was also observed in the stromal cells(Table 4). In addition, only the total PI values for Notch 3 showed statistically significant difference between functional and basal layer (Table 4)

The positive cytoplasmic staining for Notch ligands was also observed in endometrial glandular cells. Cyclic changes in the expression of these molecules during the menstrual cycle were not observed. The expression of JAG1 and DLL4 in the stromal cells was also constant throughout the menstrual cycle. The mean PI for total JAG1 and DLL4 in the glandular epithelium of the functional layer was 70.2± 6.3 and 74.0 ± 6.3, while that in the basal layer was 69.2 ± 5.4 and 74.2 ± 5.6, respectively (Table 3; Figs 1G-H, and J-K). There was no significant difference between PI values for the proliferative and secretory phases (Figs 1I and 1L).

EXPRESSION OF NOTCH LIGANDS AND NOTCH RECEPTORS IN ENDOMETRIAL CARCINOMA

The PI values for Notch receptors and ligands in relation to the different histological grades of endometrial carcinoma are summarized in Table 5. Representative images of immunostaining for Notch1, Notch3, Jag1, and DL-4 are shown in figures 2A-B, D-E, G-H, and J-K. The mean PI values for Notch1 and Notch3 in all endometrial carcinomas were increased compared with those in normal endometrial epithelium of the functional layer ($P<0.05$, Fig. 2C). The mean PI for Notch1 tended to increase according to histological grade (Fig. 2C). There was a significant difference in the PI for Notch 1 between early (stage I) and advanced (stage III/IV) stage tumors as shown in figure 2C. The mean PI for Notch3 was relatively constant among the three histological grades (Fig. 2F). In addition, it was not increased in the advanced stages compared with early stages. Immunostaining for Notch ligands was also increased in endometrial carcinomas compared with normal endometria (Fig. 2I and 2L). The mean PI for Notch ligands did not show any significant change according to histological grade or stage (Fig. 2I and 2L). In addition, there was little variation in the strength of the staining for Notch-related proteins in tumor tissue. Then we examined whether the expression of Notch receptors was associated with various clinicopathological parameters (Table 6). High Notch 1 expression was significantly associated with high FIGO stage, ovarian metastasis, myometrial invasion, and vessel involvement. In contrast, a high level of Notch 3 showed no significant association with such parameters (Table 6). Furthermore, PI for Notch 1 was significantly associated with high FIGO stage in multivariate regression analysis (Table 7).

SURVIVAL ANALYSIS

Of the 76 patients examined in the present study, all except those in FIGO stage Ia without deep myometrial invasion (>1/2) received platinum-based adjuvant chemotherapy after their surgery. None received radiation therapy after operation. Nine had died from the disease and the remaining 67 were alive with no evidence of disease at the last follow-up. The mean post-treatment follow-up period was 64.9 months. In this study, a PI value larger than the mean was considered to indicate “high” expression. The prognosis of the 76 patients with endometrial carcinoma was determined with Kaplan-Mayer overall survival curves for Notch1, Notch3, JAG1, and DLL4 (Figs. 3A-3D). The mean survival time for Notch1-low and -high cases was 109.1 (4-172) and 95.5 (9-168) months, respectively. The mean survival time was 118.2 (21-172) months for Notch3-low cases and 92.1 (4-168) months for Notch3-high cases. The Notch 1-high and Notch3-high patients had significantly poorer rates of survival than the Notch1- low and Notch3-low patients (P =0.005 and 0.041, respectively, Fig 3A and 3B). There was also a significant difference in survival between the Notch ligand-high and -low patients (Fig. 3C and 3D). We then performed a subgroup analysis based on the status of immunoreactivity for Notch-related molecules to reveal the functional association of ligands and receptors and their contribution to patient survival. Patients having tumors with high levels of both JAG1 and Notch1 had a worse overall survival rate than the patients with tumors expressing high levels of either JAG1 ($Notch1^{lo}/JAG1^{hi}$) or Notch1 ($Notch1^{hi/}JAG1^{lo}$), and low levels of both JAG1 and Notch1 ($Notch1^{lo/}JAG1^{lo}$, Fig.3E). No similar tendency was observed for other combinations (data not shown).

EFFEXT OF A γ-SECRETASE INHIBITOR ON THE PROLIFERATION OF ENDOMETRIAL CARCINOMA CELLS

The expression of Notch1, Notch3, and Notch ligands (DLL4 and JAG1) was evaluated in five endometrial carcinoma cell lines. Both Notch1 and Notch3 were detected in all

the cell lines. There was little variation in the expression of Notch1 and Notch3 among these cell lines (Fig. 4A). To study the effect of the inhibition of Notch signaling, a γ-*secretase inhibitor* (DAPT) was added to the five endometrial carcinoma cell lines and viability was measured using the WST-1 assay. No growth suppression was observed in any of the cell lines at a final concentration of $1x10^{-5}$ M (Fig. 4B).

EFFEXT OF A γ-SECRETASE INHIBITOR ON THE ADHESIVE AND INVASIVE PROPERTIES OF ENDOMETRIAL CARCINOMA CELLS

We examined the involvement of the Notch pathway in the adhesion of endometrial carcinoma cells, however, there was no significant change in the number of adherent cells after the addition of DAPT even at $1x10^{-6}$M (Fig.5A). We then tested the effects of Notch inhibition on the invasion of KLE cells using scratch wound healing and matrigel invasion assays. Cell migration was inhibited 36 hours following the treatment with DAPT (Fig. 5B). To further confirm this effect, we measured DAPT-induced invasion 48 hours after the exposure of KLE cells to DAPT. The results showed that the number of migrating cells decreased with DAPT treatment with a significant difference at 1 x 10^{-6}M (Fig. 5C and 5D).

Discussion

The present study demonstrated the expression of Notch signaling molecules in normal and malignant endometria. The expression of Notch receptors (Notch 1 and Notch3) and ligands (JAG1 and DLL4) was predominantly observed in glandular cells in the normal endometrium. No cyclic change in the expression of Notch signaling molecules during the menstrual cycle was observed except for Notch 3 in the stroma, which increased in the secretory phase. Consistent with our results, Cobellis et al. demonstrated weak immunostaining of Notch receptors in glandular cells during the proliferative phase.[19]

Notch signaling is a primitive pathway which needs cell-to-cell contact for activation. In contrast, humoral factors such as sex steroid hormones, growth factors, and cytokines can activate large amount of cells at once. Therefore, we concluded the contribution of the Notch pathway to the marked proliferative potential in normal endometrium to be limited. In the present study, we did not observed a significant increase in the expression of Notch receptors in the secretory phase, however, a previous study suggested the involvement of the Notch pathway in the differentiation of normal endometrium, in which the expression of Notch 1 was stronger in the secretory phase than proliferative phase.[19] The discrepancy with our results might be due to the different antibody used and antigen retrieval method. In the present study, we examined the specificity of each antibody using Western blotting. Mazella et al. demonstrated a significant increase in the mRNA level of DLL4 from the mid-proliferative to early secretory phase, but no such increase at the protein level.[20] Taken together, it is unlikely that the expression of Notch-related molecules is directly involved in the proliferation or differentiation of cells in the normal endometrium. Notch signaling is reportedly involved in the maintenance of progenitor cells and strong Notch 1 expression was reported in the crypt of colon epithelium.[21] Although the levels of Notch receptors showed subtle variations between basal and functional layers where the higher proliferative activity was observed, such a topological distribution in each gland was not evident.

The present study also revealed that all Notch signaling molecules were expressed more strongly in endometrial carcinoma cells than in normal endometrial glandular cells. A study demonstrated increased Notch1, and reduced JAG 1, expression in 20 endometrial carcinoma samples.[19] The difference in the expression of JAG 1 in endometrial carcinoma may be in part due to the small number of cases examined. In the present study, we examined the association of Notch signaling molecules with various clinicopathological parameters including histological grade, clinical staging,

and overall survival. The results showed that the expression of Notch 1 was increased in tumors with invasive properties such as vessel or lymph node involvement, and myometrial invasion. Consistent with this, the inhibition of Notch signaling by DAPT treatment suppressed the invasiveness and motility of endometrial carcinoma cells. These results were in line with recent studies in prostate and gastric cancers in which targeted knockdown of Notch 1 inhibited the invasion of cancer cells.[22, 23] In contrast, the adhesion of KLE cells was not affected by the disruption of Notch signaling. This is not surprising because notch signaling needs cell-to-cell contact for activation. Importantly, the present study revealed that the expression of Notch 1 was independently correlated positively with high FIGO staging and poor overall patient survival. Both JAG1 and DLL4 could bind to Notch 1, however, only a high level of JAG1/Notch 1 was significantly associated with a poor prognosis. Consistent with this, JAG1-mediated autocrinal or juxtacrinal activation of Notch signaling in aggressive cancers was proposed.[24-26] In addition, JAG1/Notch 1's activation was associated with poor overall survival in breast cancer patients.[25] The precise mechanism of Notch signaling for tumor invasion remains undetermined, however, recent studies suggested Notch1's activation to be associated with the expression of genes involved in invasion such as the genes for cyclooxygenase, metalloproteinase, snail, and vascular endothelial growth factors (VEGFs). [23,28-29] In addition, studies suggested that Notch pathways might play an important role in tumor angiogenesis[30] and selective inhibition of the DLL4 pathway resulted in tumor regression through an anti-angiogenic mechanism.[31] A recent study also suggested crosstalk between the Notch pathway and VEGF pathway.[32] We could not examine the possible involvement of the Notch pathway in angiogenesis in the tumor tissue in this study. Thus, further study is needed to clarify the contribution of Notch signaling to the progression of endometrial cancer.

We previously reported that proliferative activity was increased according to

tumor grade,[33] however, the results did not show a significant association of Notch-related molecules with histological grade. Therefore, it was not surprising that Notch inhibition with a γ-secretase inhibitor did not suppress cell proliferation in endometrial carcinoma cell lines. A study revealed that Notch inhibition reduced the proliferative as well as invasive capacity of prostate cancer cells.[22] γ-secretase inhibitor also stopped the cell cycle at the G2/M phase in estrogen receptor (ER)- negative breast cancer cells by down-regulating the expression of cyclin A2 and B1.[34] We examined endometrial carcinoma cell lines including ER-positive (Ishikawa and HHUA) and negative (Hec-1A, Hec-1B, and KLE) cells (Table 1), but observed no difference with respect to ER status. Notch signaling exhibited crosstalk with other signaling molecules involved in cell proliferation such as WNT,[35] phosphatidylinositol 3-kinase,[36] and MAPK.[37] It seems likely that the involvement of Notch signaling in proliferation depends on cell context.

There is evidence to suggest that Notch signaling plays an important role in the self-renewal and maintenance of cancer stem cells.[38] Recent studies have also revealed the existence of cells with stem cell-like properties in brain,[39] breast,[40] and ovarian[41] cancers. Notch inhibition resulted in the depletion of CD133-positive cancer stem cells in glioblastomas.[42] Some studies have identified cell subpopulation which showed stem cell-like properties in normal endometrial glandular cells[43] and endometrial cancer cells[44,45], however, the specific cell surface marker for stem cells have not been identified or established. Further studies will be required to examine the involvement of cancer stem cells in endometrial cancer.

In conclusion, the Notch 1- JAG1 axis in endometrial carcinoma cells correlated with a poor prognosis. Notch inhibition by gamma-secretase inhibitor suppressed invasion and motility in endometrial carcinoma cell lines. These results suggest Notch signaling to be a possible target in the treatment of endometrial

carcinoma. Further study is warranted.

Conflict of Interest Statement

None declared.

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Figure legends

Figure 1. Results of immunostaining for Notch1, Notch3, JAG1, and DLL4 in the normal endometrium during the proliferative (**A, D, G and J**) and secretory (**B, E, H and K**) phases. The expression of Notch receptors (**A-B and D-E**) and ligands (**G-H and J-K**) was distributed diffusely in the cytoplasm of glandular cells in both phases. The stromal cells also express these molecules but with decreased intensity. (original magnification: x 100): Graphic demonstration of the PI for Notch1 (**C**), Notch3 (**F**), JAG1(**I**), and DLL4(**L**) in the proliferative (white column) and secretory (black column) phase. A black bar beneath the panels indicates topological information on the endometrium. Each bar indicates the mean ± SD. *, significant difference ($P<0.05$) between the functional and basal layers.

Figure 2. Results of Immunostaining for Notch1 (**A** and **B**), Notch3 (**D** and **E**), JAG1 (**G** and **H**), and DLL4(**J** and **K**) in endometrial carcinomas of low (upper panel; **A,D,G,** and **J**) and high (lower panel; **B,E,H,** and **K**) grade(original magnification: x100). **C,F,I and L,** two left-most bars: Graphic demonstration of the PI for Notch1, Notch3, JAG1, and DLL4 in normal and malignant endometria. *: $P<0.05$**. M**iddle, and two right-most bars: Graphic demonstration of the PI according to histological grading and FIGO staging. Each bar indicates the mean ± SD. *, significantly different from that in normal endometrium, **, significantly different from that in early staged tumors.

Figure 3. Kaplan-Mayer overall survival analysis of patient survival with respect to high-level expression of Notch1 (**A**) , Notch3 (B), JAG1 (**C**), or DLL4 (**D**). The mean PI value for each molecule was used as the cut off to define “high” patients. Patients harboring high-level expression of Notch ligands had a significantly poorer outcome. **E**: Kaplan-Meier curve showing the relationship between high-level Notch1 + JAG1

co-expression and overall survival in patients with endometrial carcinomas.

Figure 4. **A**: Results of Western blotting for Notch1, Notch3, JAG1, and DLL4 in endometrial carcinoma cell lines. **B**: Effects of DAPT on the proliferation of the endometrial carcinoma cell lines. Viability was measured 72 hours after the addition of DAPT. Serial concentrations of DAPT were added in six independent wells. Untreated and vehicle-treated controls were included.

Figure 5. Effect of DAPT on the adhesive and invasive properties of KLE cells. **A:** The WST-1 assay to analyze the adhesion of KLE cells onto collagen type IV-coated dishes, 120 minutes after DAPT treatment. Six wells were allocated to each group. **B:** Effect of DAPT on the motility of KLE cells accessed by a wound healing assay system as described in materials and methods. In control experiments, cells were exposed to the vehicle alone. Cell migration was assessed by directly counting the number of cells migrating into the intersecting denuded area 48 hours later. *, $p<.05$. C: Representative images of the Matrigel invasion assay after inhibition of the Notch pathway by DAPT. The right panel shows that decreased numbers of KLE cells were stained at a magnification of x200. **D**: Graphic demonstration of the Matrigel invasion assay. *: significant difference ($P<0.05$).

Table 1

List of endometrial carcinoma cell lines

	Histological Grade	ER	Medium	FBS
Ishikawa	well differentiated adenocarcinoma	positive	DMEM	15%
HHUA	well differentiated adenocarcinoma	positive	F-12	15%
Hec-1A	moderately differentiated adenocarcinoma	negative	Macoys' 5a	10%
Hec-1B	moderately differentiated adenocarcinoma	negative	DMEM	10%
KLE	poorly differentiated adenocarcinoma	negative	DMEM/F-12	10%

ER: estrogen receptor

Table 2

List of antibody sources and dilutions

	Source	City	ID	Isotype	Staining dilutions
Notch 1	Santa Cruz Biotechnology	Santa Cruz, CA	sc-6014R	Rabbit	1:50
Notch 3	Santa Cruz Biotechnology	Santa Cruz, CA	sc-5593	Rabbit	1:50
Jag-1	R&D systems	Minneapolis, MN	AF1277	Goat	1:100
Delta-4	Rockland Immunochemicals	Gilbertsville, PA	600-401-696	Rabbit	1:100

Table 3

Results of immunostaining for Notch1, Notch 3, and Notch ligands in normal endometrial glands

	Functional layer			Basal layer		
	Phase		Total	Phase		Total
	proliferative n=19	secretory n=18	n=37	proliferative n=19	secretory n=18	n=37
Notch1 PI	72.1±10.2	71.8±8.5	71.9±9.2	71.0 ±17.4	67.5±10.0	69.3±14.1
Notch3 PI	75.0 ±6.0	77.2±8.9	76.1±7.6	72.1±7.1	74.4±7.8	73.2±7.5
Jagged1 PI	69.8±6.4	70.6±6.4	70.2±6.3	69.4±5.2	69.0±5.7	69.2±5.4
Delta4 PI	73.0±4.3	75.2±8.2	74.0±6.3	74.0±4.0	74.5±7.2	74.2±5.6

Each PI value is indicated as the mean ± SD.

Table 4

Results of immunostaining for Notch1, Notch3, and Notch ligands in normal endometrial stroma

	Functional layer			Basal layer		
	Phase		Total	Phase		Total
	proliferative n=19	secretory n=18	n=37	proliferative n=19	secretory n=18	n=37
Notch1 PI	30.0±5.9	31.4±6.6	30.7±6.2	23.3±4.9	22.8±4.3	23.1±4.5
Notch3 PI	30.4±11.7	37.2±14.6	33.7±13.4	22.7±4.3	23.9±5.0	23.3±4.6*
Jagged1 PI	32.1±7.3	26.8±5.8	29.7±7.1	21.8±3.9*	22.5±4.3	22.1±4.0
Delta4 PI	30.6±6.6	29.0±6.4	29.9±6.4	21.8±3.9	22.3±4.4	22.0±4.1

Each PI value is indicated as the mean ± SD.

*Significantly different from the functional layer ($P<0.05$)

Table 5

Results of immunostaining for Notch1, Notch3, and Notch ligands in malignant endometria

	Histological grade			Stage			Total
	1	2	3	I	III+IV		
	n=50	n=9	n=17	n=57	n=19		n=76
Notch1 PI	88.6±13.7	92.5±17.0	94.0±18.5	87.4±13.5	97.7±17.2	**	90.4±15.3***
Notch3 PI	100.9±19.3	82.0±18.7	88.9±17.8	96.3±20.7	93.9±18.6		95.6±20.0***
Jagged1 PI	95.6±11.0	92.2±7.5	97.3±8.2	94.4±10.3	98.2±9.0		95.5±10.0***
Delta4 PI	88.0±8.2	89.3±8.9	87.3±12.7*	88.2±8.7	88.3±11.9		88.2±9.6***

NOTE: Each PI value is indicated as the mean ± SD.

* Significantly different from grade 1 ($P<0.05$)

** Significantly different from stage I ($P<0.05$)

*** Significantly different from normal endometrial gland (functional layer) ($P<0.05$)

Table 6

Association of tumors having high-level Notch1 and Notch3 expression with clinicopathologic parameters

Variable	Notch 1			Notch3	
	χ^2	p		χ^2	p
Grade	1.77	0.412		3.77	0.152
Stage(I vs. III,IV)	3.84	0.047	*	0.32	0.858
Deep myometerial invasion (<1/2 v.s >1/2)	16.35	0.001	*	0.64	0.968
Vessel involvement	2.56	0.011	*	0.39	0.842
Cervical invasion	1.41	0.235		0.62	0.43
Nodal status	2.29	0.13		0.31	0.58
Ascites	0.294	0.587		0.11	0.746
Ovarian metastasis	4.95	0.026	*	0.003	0.959

*p<.05

Table 7. Multivariate Regression Analysis of the Relationship BetweenNotch1 and FIGO Stage (as an outcome variable)

Variables in the Final Model	β	95% CI	*P*
Tumor grade	0.138	0.034-0.104	<0.001
Lymph node metastasis	0.844	0.786-0.949	<0.001
Ovarian metastasis	0.172	0.148-0.341	<0.001
Vessel involvement	-0.93	-0.142- -0.015	0.016
PI value for Notch 1	0.067	0.001-0.003	0.027

CI: Confidence interval

Figure 1

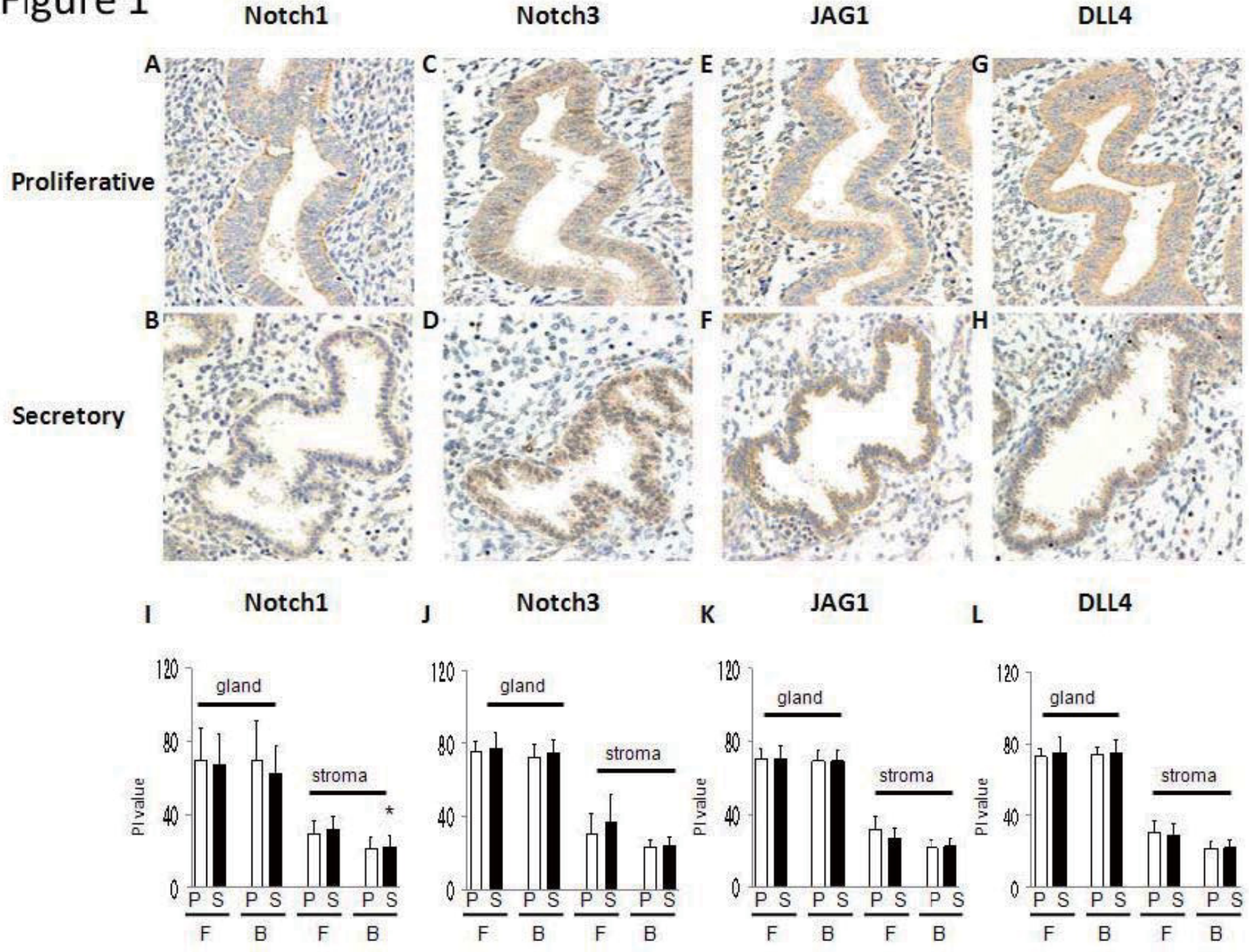

Notch1
Notch3
JAG1
DLL4
A
C
E
G
Proliferative
B
D
F
H
Secretory
I
Notch1
J
Notch3
K
JAG1
L
DLL4
PI value
120
80
40
0
gland
stroma
*
P S P S P S P S
F B F B

Figure 2

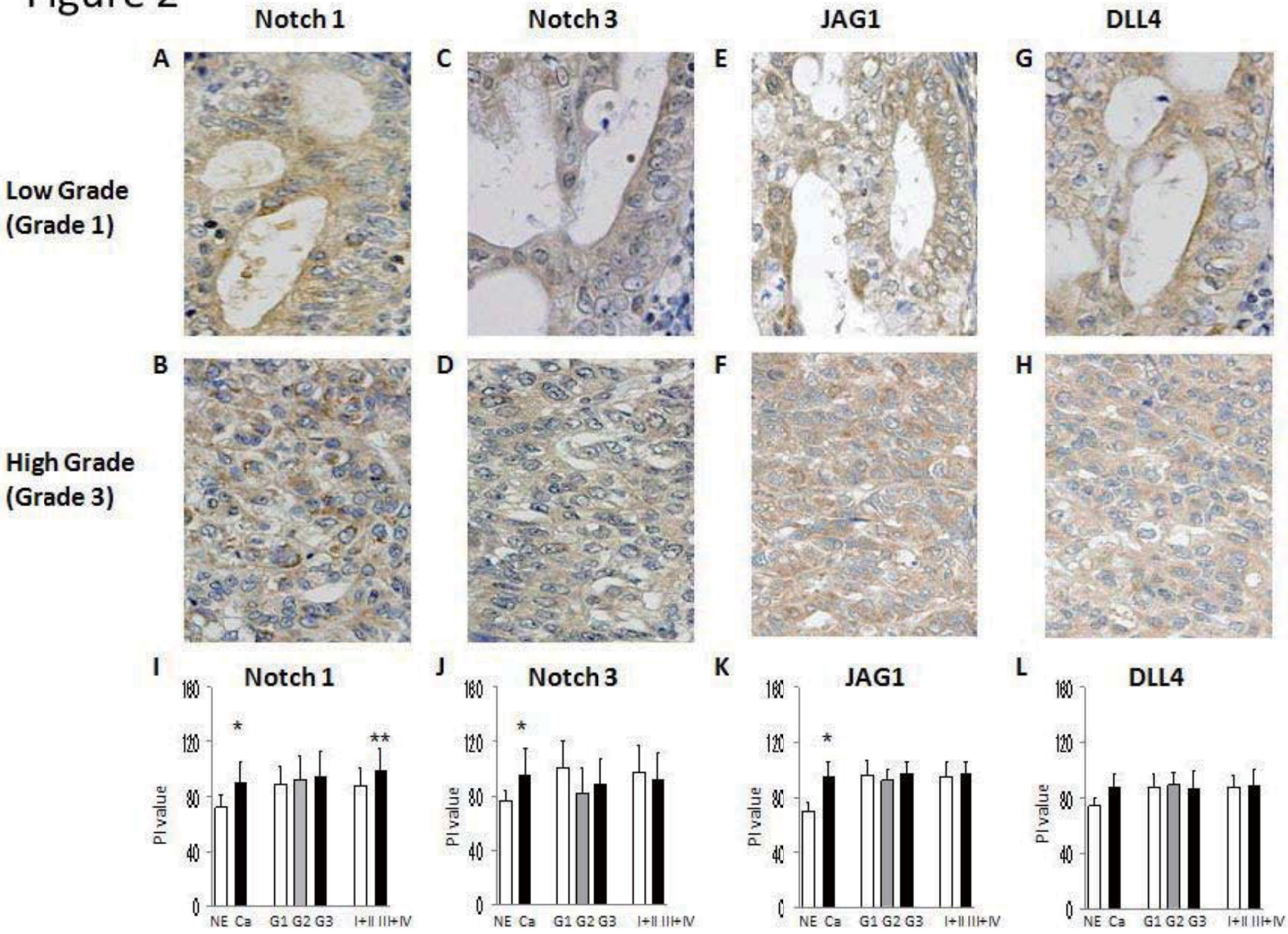

Notch 1
Notch 3
JAG1
DLL4
A
C
E
G
Low Grade
(Grade 1)
B
D
F
H
High Grade
(Grade 3)
I
Notch 1
J
Notch 3
K
JAG1
L
DLL4
PI value
160
120
80
40
0
*
**
NE Ca G1 G2 G3 I+II III+IV

Figure 3

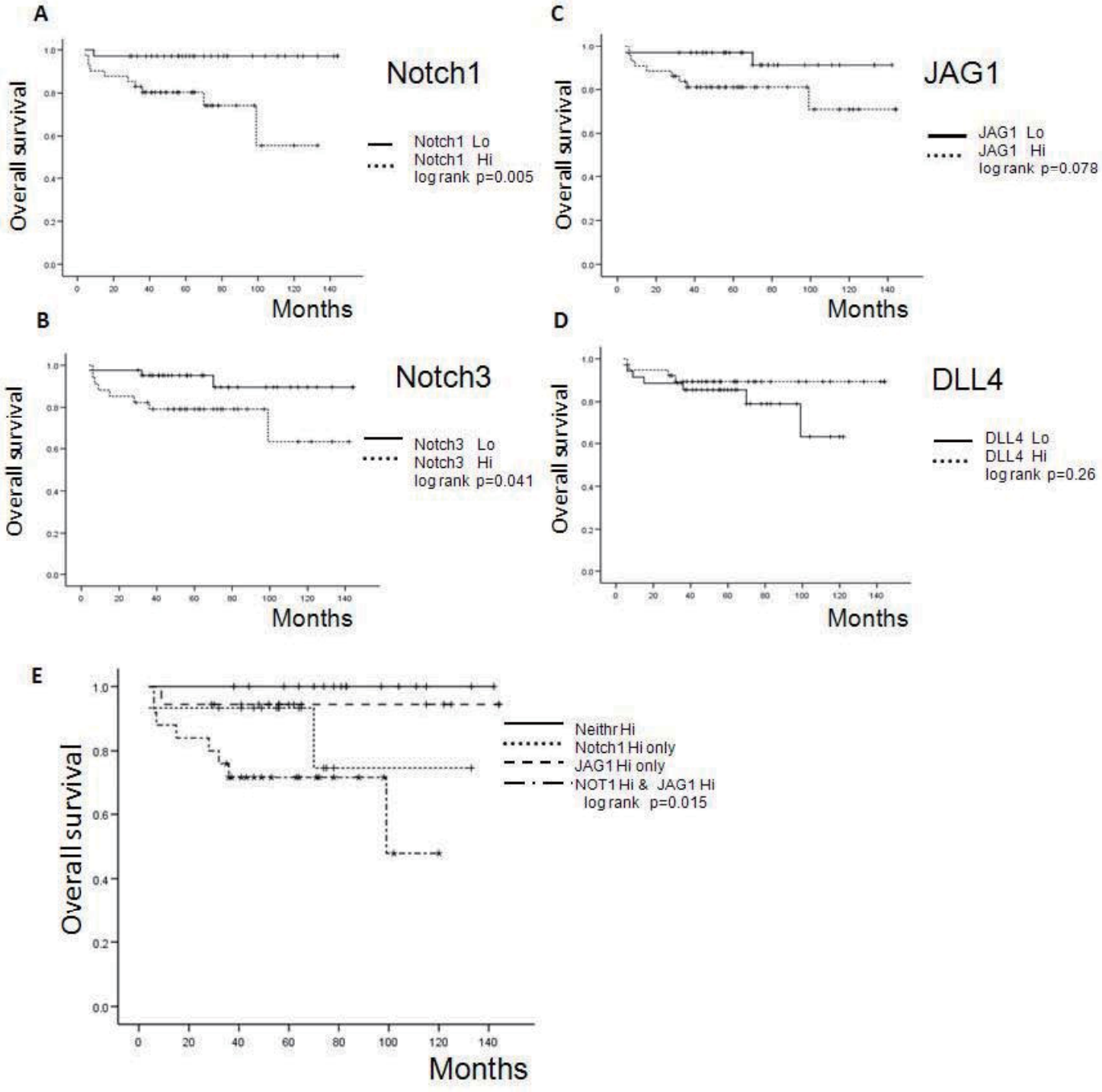
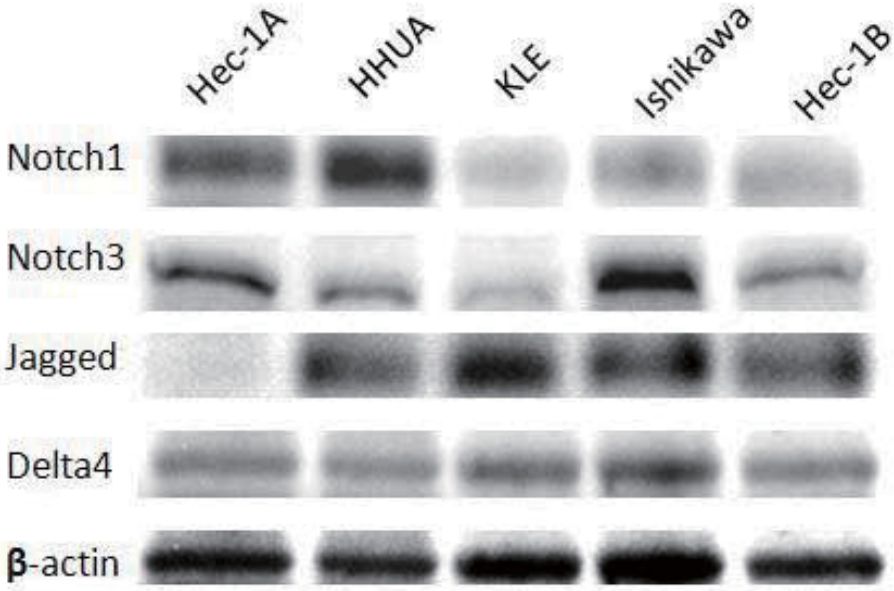

A
Notch1
Overall survival
Months
Notch1 Lo
Notch1 Hi
log rank p=0.005
B
Notch3
Overall survival
Months
Notch3 Lo
Notch3 Hi
log rank p=0.041
C
JAG1
Overall survival
Months
JAG1 Lo
JAG1 Hi
log rank p=0.078
D
DLL4
Overall survival
Months
DLL4 Lo
DLL4 Hi
log rank p=0.26
E
Overall survival
Months
Neithr Hi
Notch1 Hi only
JAG1 Hi only
NOT1 Hi & JAG1 Hi
log rank p=0.015

Figure 4

A


Hec-1A
HHUA
KLE
Ishikawa
Hec-1B
Notch1
Notch3
Jagged
Delta4
β-actin

B

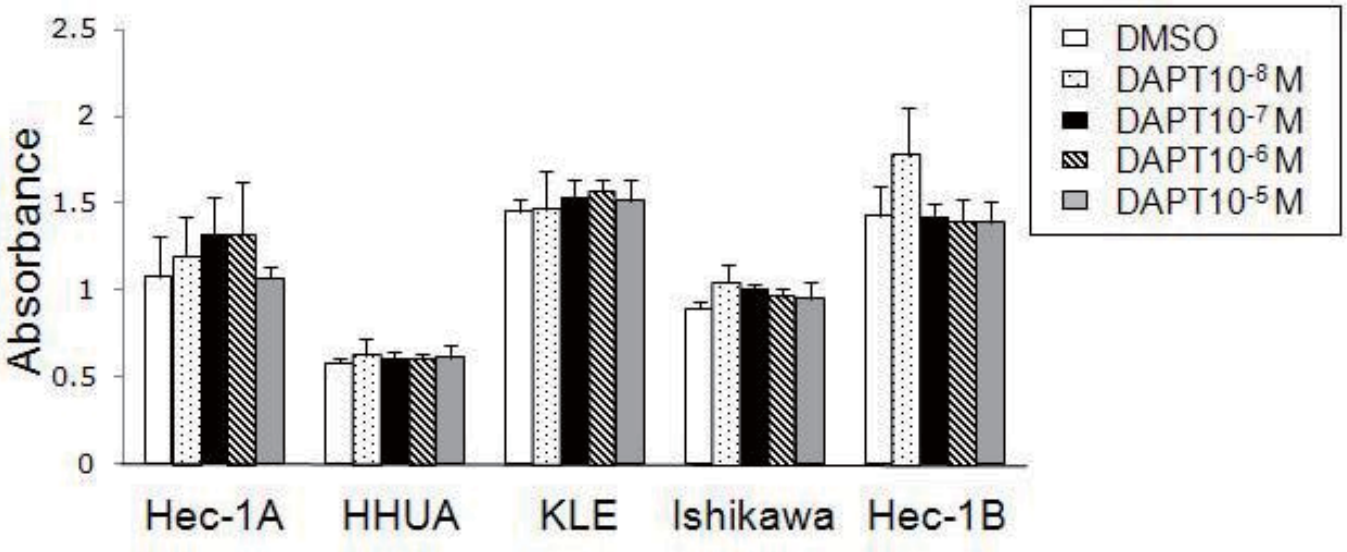
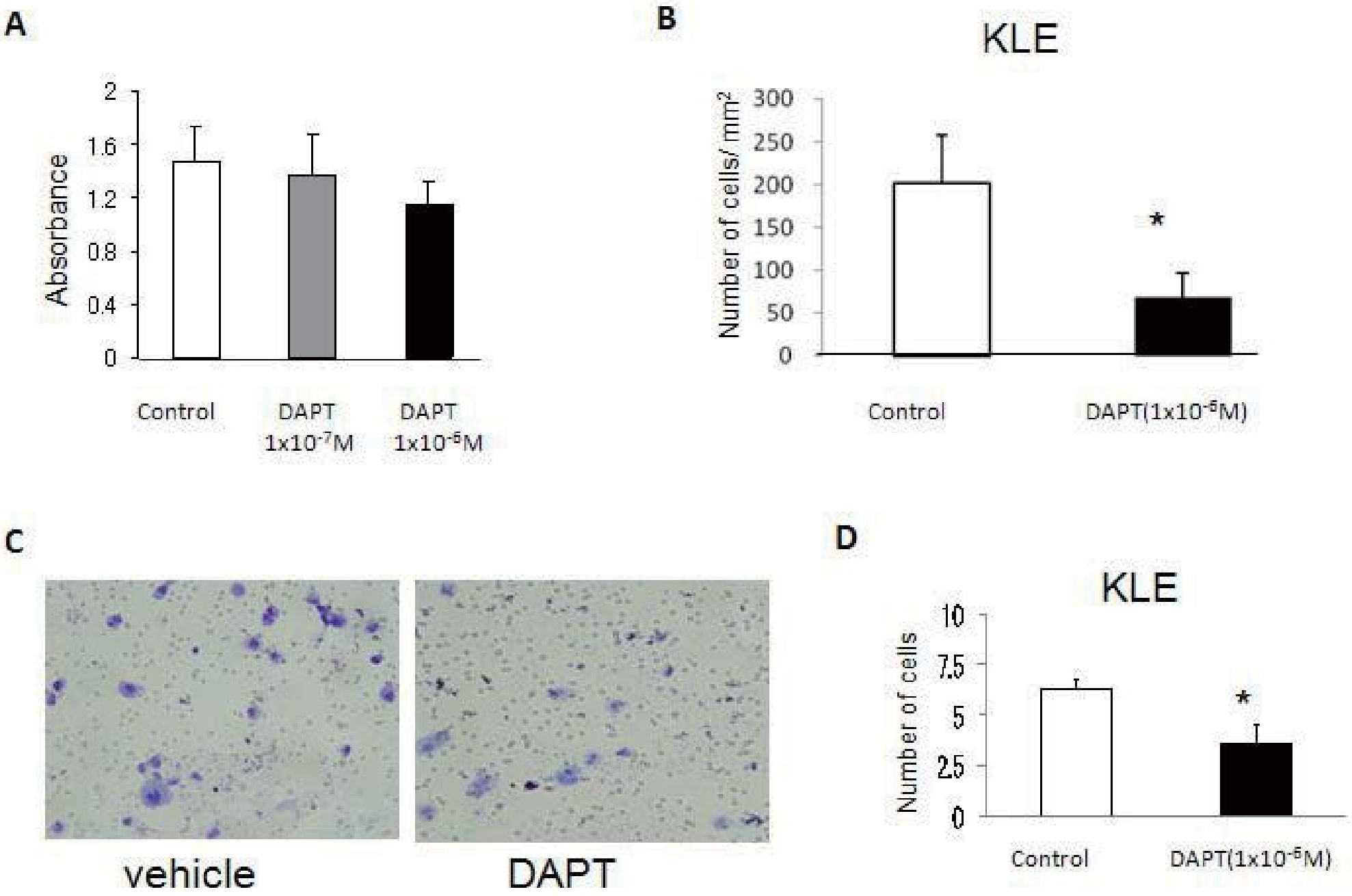

Absorbance
2.5
2
1.5
1
0.5
0
Hec-1A
HHUA
KLE
Ishikawa
Hec-1B
DMSO
DAPT10-8 M
DAPT10-7 M
DAPT10-6 M
DAPT10-5 M

Figure 5


A
Absorbance
2
1.6
1.2
0.8
0.4
0
Control
DAPT 1x10-7M
DAPT 1x10-6M
B
KLE
Number of cells/ mm2
300
250
200
150
100
50
0
*
Control
DAPT(1x10-6M)
C
vehicle
DAPT
D
KLE
Number of cells
10
7.5
5
2.5
0
*
Control
DAPT(1x10-6M)