# PHLDA1 expression in ulcerative colitis: A potential role in the management of dysplasia

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**Abstract.** Pleckstrin homology-like domain, family A, member 1 (PHLDA1) is a protein involved in cell proliferation, adhesion and migration in colon cancer. In normal large intestinal mucosa, this protein is expressed only in the crypts. By contrast, its expression in adenomas and cancers of the large intestine is spread throughout the glandular ducts, and it has been reported that PHLDA1 may be involved in the process of carcinogenesis. PHLDA1 may also be involved in the pathogenesis of ulcerative colitis (UC). The expression levels of PHLDA1 in tissues from patients with UC were analyzed using immunohistochemistry, and its relationship with the development of UC-associated colorectal cancer (UC-CRC) was examined. Overall, tissue samples from 143 lesions (90 colitis lesions, 39 dysplastic lesions and 14 UC-CRC lesions) were prepared from excised specimens of 49 patients with UC who underwent surgery in Tokyo Medical and Dental University Hospital between January 2004 and December 2017. Subsequently, immunostaining for PHLDA1 was performed. PHLDA1 expression was evaluated in UC-CRC and dysplastic tissues within the entire lesion area on the slide and in colitis over the area of the accompanying duct. The cytoplasmic staining intensity was classified into four levels, and the expression score (0-2 points) was calculated. The median PHLDA1 expression score was 0.295 for colitis, 0.607 for dysplasia and 0.865 for UC-CRC. The dysplasia expression score was significantly higher than the colitis score (P<0.001), while the UC-CRC expression score was significantly higher than the dysplasia score (P=0.003). The expression levels of PHLDA1 in UC cases were higher in colitis, followed by dysplasia and UC-CRC, which suggested that this protein may be involved in the carcinogenesis of UC-CRC. In addition, PHLDA1 immunostaining may help in the diagnosis of dysplasia, which is a type of precancerous lesion.

## Introduction

The worldwide incidence of ulcerative colitis (UC) continues to increase at a significant rate (1). UC is a type of inflammatory bowel disease characterized by periods of inflammatory recurrence and remission events, which are accompanied by cell death and regeneration of the colonic mucosa. It is well known that long-standing UC leads to dysplasia (i.e., precancerous lesions) and colorectal cancer (CRC) and is often a threat to the lives of the patients. The incidence of colorectal dysplasia in patients with UC has been reported to be 1.9% at 5 years, 5.1% at 15 years, and 9.2% at 25 years after the onset of UC (2). The risk of developing UC-associated CRC (UC-CRC) increases 0.5-1% per year in patients who have had UC for longer than 8-10 years (3). In addition, the prognosis of CRC is generally poorer in patients with UC than in patients without UC (4). Therefore, surveillance colonoscopy is recommended for the detection of neoplasms in patients with UC. Early detection of UC-CRC is essential for the successful management of long-standing UC (4). However, endoscopic and histologic detection of dysplasia is often difficult due to the presence of inflammatory and subsequent regenerative changes in the colonic mucosa (3,5). For the early detection of dysplasia, it is important to understand the mechanism of CRC development in patients with UC. While chronic inflammation of the colonic mucosa is believed to cause UC-CRC (6), the genetic details of UC-CRC pathogenesis remain unclear (7). In cases of CRC in patients without UC, p53 mutations are generally considered to be involved in the later stages of carcinogenesis (8), while in UC-CRC, p53 mutations have been reported to occur earlier in tumor development (3,7). p53 immunohistochemistry (IHC) is therefore often used for the diagnosis of neoplasms in UC.

In both non-UC-CRC and UC-CRC, the expressions of some genes have different effects on carcinogenesis, but these two diseases also share many common genes (9).

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Previously, our laboratory reported 17 genes associated with distant metastases extracted from microarrays using gene expression data (10), as well as the involvement of the *ATF6* in the carcinogenic process of UC (11). ATF6 was rarely expressed in normal mucosa but highly expressed in colon adenomas and CRC. This gene was confirmed to be highly expressed in dysplasia lesions and UC-related cancers. Therefore, we searched for genes that may be involved from the early stages of canceration to the metastatic stage and selected *PHLDA1*, which encodes for pleckstrin homology-like domain, family A, member 1, which is one of the 17 genes (12). In this study, we focused on the possible involvement of *PHLDA1* in UC carcinogenesis and cancer progression.

**Materials and methods**

*Identification of PHLDA1*. The microarray data used was obtained from a previous study (13). The gene expression data are deposited in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE32323.

The gene expression data were analyzed to identify the candidate genes related to distant recurrence. The criteria to select candidate genes were as follows: i) A higher expression level in cancer tissues than in non-cancerous mucosa, and ii) a significantly higher expression level in cancer cells from the recurrence group than in the non-recurrence group. A higher expression was defined as a numerical value 1.5 times greater than those in another group (10). In the microarray analysis, 69 genes were identified that fulfilled the abovementioned criteria. Among 69 genes, 17 genes were found associated with human malignancies. Among the 17 candidate genes, we focused on *PHLDA1*, which is expressed in adenomas in the early stages of canceration and is also involved in cancer progression.

*Patients and samples for the IHC study*. A total of 49 consecutive patients with UC who underwent colectomy between January 2004 and December 2017 were included in this study. Overall, 143 lesions were analyzed in the IHC study. The diagnosis was based on surgically resected UC specimens and was made by pathologists who specialize in colon pathology. The pathologic findings of the UC samples were categorized into two groups: The absence of neoplasia group (colitis) and the neoplasia group (dysplasia and UC-CRC).

Samples of non-neoplastic lesions from patients with or without neoplasia were also selected. Samples of neoplasia were selected from each neoplastic lesion present. Neoplastic and non-neoplastic lesions comprised one sample from a single lesion. Table I summarizes the patients' characteristics.

*Immunohistochemistry*. IHC analysis was performed on 4-$\mu$m-thick sections cut from formalin-fixed paraffin-embedded tissue blocks obtained from each patient. PHLDA1 and p53-IHC analysis was performed on all samples in this study. All sections were scored independently by two investigators.

*IHC for PHLDA1*. The streptavidin-biotin method was used for PHLDA1 immunostaining. Xylene is soaked for 10 min five times. In addition, it is soaked in 100% ethanol three times for 5 min, 95% ethanol once for 3 min, 90% ethanol once for 3 min, 80% ethanol once for 3 min, and 70% ethanol once for 3 min. Then it is rehydrated with double-distilled water for 5 min three times.

Antigen retrieval was performed by heating the tissue sections in pH 6.0 citrate buffer in a microwave at 98˚C for 25 min. Then, sections were then incubated in a solution of 3% hydrogen peroxide in 100% methanol for 15 min at room temperature to quench endogenous peroxidase activity. Next, sections were incubated in a solution of rabbit polyclonal antibody against PHLDA1 (1:250; sc-23866; Santa Cruz Biotechnology) for 15 min at room temperature and then for a further 16 h at 4˚C, after which they were labeled with a polymer [Histofine Simple Stain MAXPO (MULTI); Nichirei Bioscience] for 30 min at room temperature. Staining was visualized after incubation with DAB (0.02% 3,3'-diaminobenzidine tetrahydrochloride; Nichirei Bioscience) for 10 min at room temperature. Finally, the slides were counterstained in 1% Mayer's hematoxylin, after which they were dehydrated in a series of increasing alcohol concentrations, which was followed by xylene immersion, mounting, and coverslipping.

PHLDA1-IHC was evaluated according to a modification of the method previously described by Krajewska *et al* (14). Positive cytoplasmic staining in colitis, dysplastic, and UC-CRC cells was assessed and scored on the basis of the immunostaining intensity. The cytoplasmic immunostaining intensity of the ductal cells was graded as - (negative), ± (weak), 1+ (strong), or 2+ (very strong) compared with the stroma cells. The numbers of cells that exhibited each grade of staining intensity were counted independently and totaled. Scores corresponding to the percentage (%) of cells of each grade of staining intensity were calculated relative to the total number of cells. The score of cytoplasmic staining for PHLDA1 (potentially ranging from 0 to 2) was obtained by summing the product of each percentage score by the corresponding intensity score.

*IHC for p53*. The streptavidin-biotin method was used for mutated p53 immunostaining. Antigen retrieval was performed by autoclaving the tissues in pH 6.0 citrate buffer at 121˚C for 15 min.

Endogenous peroxidase activity was quenched using the same method used for PHLDA1-IHC. The sections were sequentially incubated with a polyclonal antihuman p53 antibody (1:200; NCL-L-p53-DO7; Leica Biosystems) for 60 min at room temperature, MULTI for 30 min at room temperature, DAB for color development, and 1% Mayer's hematoxylin for the counterstain. Then, the sections were dehydrated by immersion in a series of alcohol solutions and xylene according to the method used for PHLDA1-IHC. As Shigaki *et al* (15) reported, the p53 staining pattern in the nucleus was characterized as sporadic, mosaic, nested, and diffuse. Nested or diffuse patterns were considered to represent p53-IHC positivity regardless of the intensity, whereas the sporadic and mosaic patterns were considered to represent p53-IHC negativity.

*Statistical analysis*. All statistical analyses were performed using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). More precisely, it is a modified version of R commander designed to add statistical functions frequently used in biostatistics. For

Table I. Characteristics of patients with UC.

| Characteristics | All patients (n=49) | Patients without neoplasia (n=30) | Patients with neoplasia (n=19) |
| --- | --- | --- | --- |
| Age, years (mean ± SD) | 47.6±16.9 | 47.7±19.5 | 47.5±11.2 |
| Sex, n (%) | | | |
| Male | 30 (61.2) | 17 (56.7) | 13 (68.4) |
| Female | 19 (38.8) | 13 (43.3) | 6 (31.6) |
| Disease extent, n (%) | | | |
| Pancolitis | 49 (100.0) | 30 (61.2) | 19 (38.8) |
| Left-sided colitis | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Others | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Past history of 5-ASA use, n (%) | | | |
| Yes | 23 (46.9) | 11 (47.8) | 12 (52.2) |
| No | 26 (53.1) | 19 (73.1) | 7 (26.9) |
| Past history of steroid use, n (%) | | | |
| Yes | 32 (65.3) | 24 (75.0) | 8 (25.0) |
| No | 17 (34.7) | 6 (35.3) | 11 (64.7) |
| Number of samples, n (%) | | | |
| Colitis | 90 (62.9) | 64 (71.1) | 26 (28.9) |
| Dysplasia | 39 (27.3) | 0 (0.0) | 39 (100.0) |
| UC-CRC | 14 (9.8) | 0 (0.0) | 14 (100.0) |

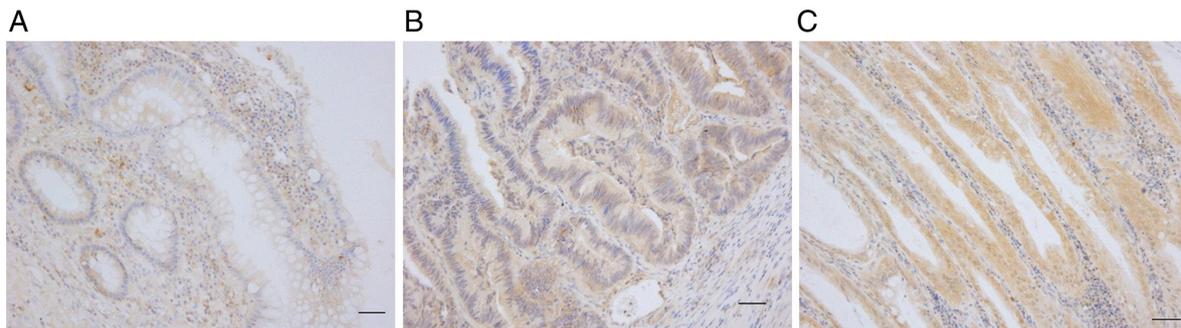UC, ulcerative colitis; 5-ASA, 5-aminosalicylate; UC-CRC, UC-associated colorectal cancer.



Figure 1. Pleckstrin homology-like domain, family A, member 1 expression in UC samples. (A) In colitis, the cytoplasmic staining of ductal cells was poor or equivalent to the staining of stromal cells. The intensities of these cells were evaluated as - or ±, respectively. (B) In dysplasia, the cytoplasmic staining of ductal cells was stronger than the stromal cell staining. The intensities of these cells were evaluated as +. (C) In UC-associated colorectal cancer, the cytoplasmic staining of ductal cells was strongly or markedly greater than that of stromal cells. The intensities of these cells were evaluated as + or 2+, respectively. Scale bar, 50 $\mu$m. UC, ulcerative colitis.

categorical data, the significance of between-group differences was estimated using the Fisher's exact test and $\chi^2$ test, as appropriate. For continuous variables, descriptive statistics of the mean, median, and range were calculated, and the significance of between-group differences was estimated using the Kruskal-Wallis test as appropriate. The Holm method was used to correct for significant differences during multiple comparisons. A P value <0.05 was considered statistically significant.

**Results**

*PHLDA1 expression in UC samples.* Of the 49 subjects, 30 exhibited no neoplastic lesions and 19 had neoplasia. The pathologies of the 143 lesions were as follows: 90 colitis,

39 dysplasia, and 14 UC-CRC. The representative results of the PHLDA1-IHC staining in UC samples are shown in Fig. 1. The median PHLDA1-IHC score of the 143 UC samples was 0.40 (range, 0.00-1.43). The median PHLDA1-IHC score in the 39 dysplasia samples was 0.607 (range, 0.03-1.32), which was significantly higher (P<0.001) than that in the 90 colitis samples (median, 0.29, range, 0-1.29). The median PHLDA1-IHC score in the 14 UC-CRC samples was 0.865 (range, 0.45-1.42), which was significantly higher (P=0.003) than that in the dysplasia samples. The PHLDA1-IHC score tended to increase as the UC cell variant progressed (Fig. 2).

*PHLDA1-IHC and p53-IHC positivity rates in UC samples.* The PHLDA1-IHC scores were divided into two groups
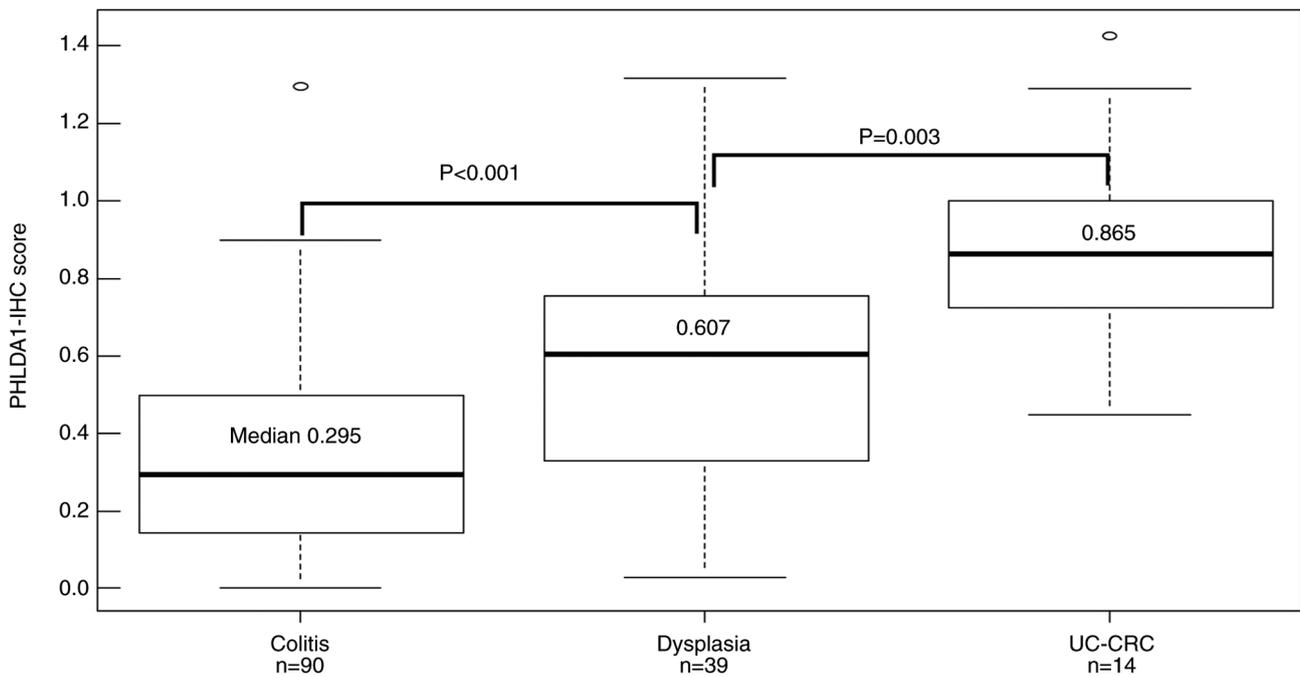
Figure 2. PHLDA1-IHC scores of the UC samples according to histological category. The median PHLDA1-IHC score of the 39 dysplasia samples was 0.607 (range, 0.03-1.32), which was significantly higher (P<0.001) than that of the 90 colitis samples (median, 0.295; range, 0-1.29). The median PHLDA1-IHC score of the 14 UC-CRC samples was 0.865 (range, 0.45-1.42), which was significantly higher (P=0.003) than that of the dysplasia samples. The Kruskal-Wallis test was performed to compare these three groups. The Holm method was used to correct for significant differences during multiple comparisons. PHLDA1, pleckstrin homology-like domain, family A, member 1; IHC, immunohistochemistry; UC, ulcerative colitis; UC-CRC, UC-associated colorectal cancer.

as follows: the PHLDA1-positive (PHLDA1-IHC score ≥0.603 n=47) and PHLDA1-negative groups (PHLDA1-IHC score ≤0.603, n=96). The cutoff value using the receiver operating characteristic curve calculated from colitis and dysplasia was 0.603, which is the threshold at which the sum of sensitivity and specificity is maximized (Fig. 3). The status of p53-IHC was classified as either p53-positive (n=25) or p53-negative (n=118) according to the method reported by Shigaki *et al* (15). The PHLDA1 and p53 positivity rates in the UC samples are shown in Fig. 4. The PHLDA1 positivity rates in neoplastic tissues were higher than that in non-neoplastic tissues. Notably, an obvious difference was observed in the PHLDA1-IHC positivity rates between UC-CRC (78.6%) and dysplasia samples (53.8%, P<0.001) and between UC-CRC and colitis samples (15.6%, P<0.001). On the contrary, the positivity of PHLDA1 was not statistically significant (P=0.067) than that of p53 in dysplasia samples.

*Accuracy of PHLDA1 and p53-IHC for a diagnosis of dysplasia.* In 129 samples including dysplasia (n=39) and colitis samples (n=90), we evaluated the accuracy of PHLDA1- and p53-IHC for the discrimination of dysplasia from the inflammatory mucosa of UC. The positive predictive value (PPV) of PHLDA1-IHC was 60.0%, which was clearly lower than that of p53 (70.6%). The negative predictive value (NPV) of PHLDA1-IHC (80.9%) was relatively higher than that of p53 (75.9%) (Table II). The sensitivity of PHLDA1-IHC was 53.8%, which was clearly higher than that of p53 (30.8%). The specificity of PHLDA1-IHC (84.4%) was relatively lower than that of p53 (94.4%). Twenty-five (17.5%) lesions were PHLDA1 positive and p53 negative, of which 14 were dysplasia (56.0%). Seven (4.9%) lesions were PHLDA1 nega-
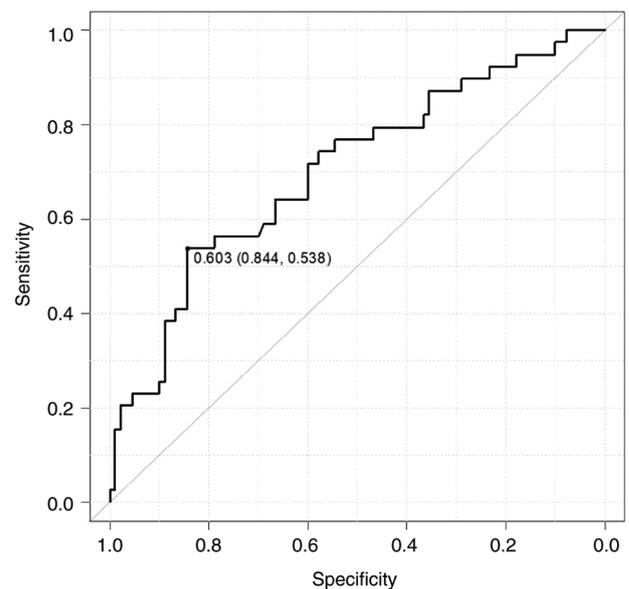


Figure 3. ROC curve. The cutoff value for colitis and dysplasia calculated using the ROC curve was 0.603 (specificity, 0.844; sensitivity, 0.538). ROC, receiver operating characteristic.

tive and p53 positive, of which five were dysplasia (71.4%) (Table III). No significant differences were observed between the PHLDA1 positive and p53 negative group and PHLDA1 negative and p53 positive group in age (P=0.65), gender (P=0.64), location (proximal/distal; P=1.00), and reason for surgery (P=1.00). The UC lesions were divided into the proximal (cecum, ascending colon and transverse colon) and distal parts (descending colon, sigmoid colon and rectum). The
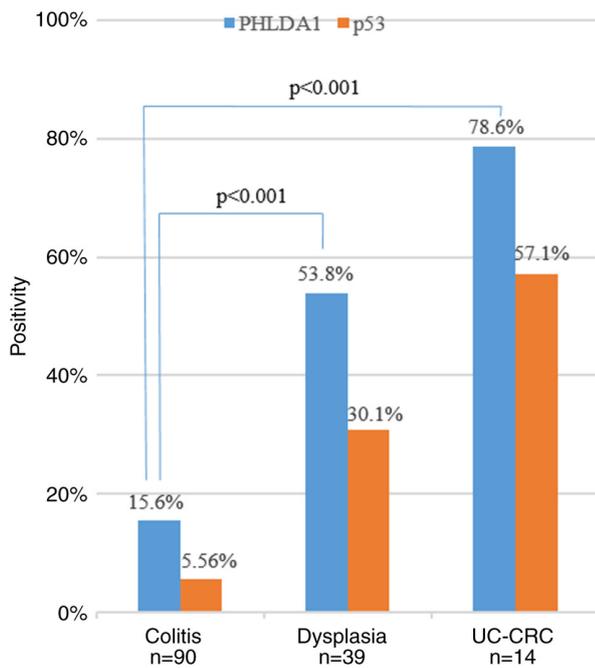
Figure 4. PHLDA1-IHC and p53-IHC positivity rates according to the histological status. The PHLDA1 positivity rate in the neoplastic tissues was higher than that in the non-neoplastic tissues. Notably, an obvious difference was observed in the PHLDA1-IHC positivity rate between the UC-CRC (78.6%) and dysplasia samples (53.8%; P<0.001), and between the UC-CRC and colitis samples (15.6%; P<0.001). Fisher's test was used to compare the ratio differences between the two groups. PHLDA1, pleckstrin homology-like domain, family A, member 1; IHC, immunohistochemistry; UC-CRC, ulcerative colitis-associated colorectal cancer.

Table II. Accuracy of PHLDA1-IHC and p53-IHC for diagnosis of dysplasia.

| Outcome | Dysplasia, n | Colitis, n | PPV, % | NPV, % |
|---|---|---|---|---|
| PHLDA1 | | | | |
| Positive | 21 | 14 | 60.0 | 80.9 |
| Negative | 18 | 76 | | |
| p53 | | | | |
| Positive | 12 | 5 | 70.6 | 75.9 |
| Negative | 27 | 85 | | |

PHLDA1, pleckstrin homology-like domain, family A, member 1; IHC, immunohistochemistry; PPV, positive predictive value; NPV, negative predictive value.

Table III. Dysplasia rate and combination of PHLDA1-IHC and p53-IHC status.

| Outcome | p53 positive | p53 negative |
|---|---|---|
| PHLDA1 positive | 70.0% (7/10) | 56.0% (14/25) |
| PHLDA1 negative | 71.4% (5/7) | 14.9% (13/87) |

PHLDA1, pleckstrin homology-like domain, family A, member 1; IHC, immunohistochemistry.

proximal parts included 53 lesions, of which 44 were colitis, and 9 were dysplasia. Ninety lesions were found in the distant parts, of which 46 were colitis, 30 were dysplasia, and 14 were UC-CRC. PHLDA1 and p53 had no significant differences in positivity rate in dysplasia in the proximal and distal regions (Table SI). On the other hand, in colitis, PHLDA1 showed a significant difference in positivity rate (Table SII). In the sites in the proximal region, the PPV of PHLDA1-IHC was 77.8%, which was clearly higher than that of p53 (66.6%). The NPV of PHLDA1-IHC (95.5%) was relatively higher than that of p53 (86.0%) (Table SIII). The sensitivity of PHLDA1-IHC was 77.8%, which was clearly higher than that of p53 (22.2%). The specificity of PHLDA1-IHC (95.5%) was lower than that of p53 (97.7%). In the sites in the distal region, the PPV of PHLDA1-IHC was 53.8%, which was clearly lower than that of p53 (71.4%). The NPV of PHLDA1-IHC (68.0%) was higher than that of p53 (67.7%) (Table SIV). The sensitivity of PHLDA1-IHC was 46.7%, which was clearly higher than that of p53 (33.3%). The specificity of PHLDA1-IHC (73.9%) was relatively lower than that of p53 (91.3%). We found no significant differences in the positivity rates of PHLDA1 and p53 between the colitis part of the patients with UC with dysplasia and/or UC-CRC (P=0.24) and those without dysplasia and UC-CRC (P=0.18) (Table SV).

**Discussion**

In this study, we demonstrated that PHLDA1 is highly expressed in dysplastic and UC-CRC lesions.

PHLDA1 was first identified as a potential transcription factor that is required for Fas expression and activation-induced apoptosis in mouse T cell hybridomas (1). The *PHLDA1* is located on 12q21.2 and contains PHL domains; these domains interact with membrane components, which elicit a variety of cellular responses, through which it participates in cell signaling transduction, vesicular trafficking, and cytoskeletal rearrangement (16,17). PHLDA1 is predominantly expressed in the cytoplasm (18-21), and its expression is induced by different stimuli, such as estrogens (22), growth factors (23), differentiation factors (24), and endoplasmic reticulum stress-inducing agents (25). PHLDA1 is also associated with various biological processes, such as cell apoptosis, cell proliferation, and differentiation.

In terms of the relationship between PHLDA1 and cancer, it has been reported that an osteosarcoma cell line with a high metastatic potential had increased PHLDA1 expression (26). It has also been found that PHLDA1 has an apoptosis-suppressing effect in oral cancer (27,28). In addition, the relationship between breast cancer and PHLDA1 has been well studied, and E2 and TNF-α are considered promoters of PHLDA1. However, *PHLDA1* in breast cancer is a tumor suppressor gene (22).

As for the relationship between *PHLDA1* and the colonic mucosa, *PHLDA1* is only expressed in undifferentiated basal cells in the crypts. It is also highly expressed in colorectal adenoma and carcinoma cells, and high expression of PHLDA1 has been shown to increase migration ability, anchorage-independent growth, and cell-matrix adhesion ability (12).

In this study, we showed that the PHLDA1-IHC score is significantly elevated as the UC cell variant progresses. Since

no studies have been retrieved showing that the canceration of UC involves the proliferation of undifferentiated basal cells, *PHLDA1* was presumed to also be involved in the canceration of UC. However, this study could not clarify whether this gene was an initiating factor or a promoting factor in the canceration of UC. Furthermore, the presence of p53 gene mutation suggests the presence of dysplasia, a precancerous lesion.

p53-IHC is widely used as an auxiliary diagnostic method, but the correct diagnosis rate of dysplasia is low, and the sensitivity is believed to be 11-40% (29), which was similar to the findings in this study. The diagnosis of high p53 protein expression by IHC does not always correspond to the p53 gene mutation. In other words, p53 IHC alone may overlook dysplasia. The detection of promising biomarkers for the diagnosis of dysplasia could impact the clinical management of patients with UC, who are at a higher risk for cancer development (3). This is because if high-grade dysplasia develops, those patients will be treated by total colectomy, which may significantly reduce the patient's quality of life. Since p53 IHC has a high PPV (70.6%), it is probable that dysplasia can be diagnosed in positive cases. Moreover, since the specificity of p53 IHC is as high as 94.4%, it can be reasonably used as an auxiliary diagnosis. However, due to its low sensitivity (30.8%), many cases of dysplasia were present among those who were diagnosed as negative. Since PHLDA1-IHC is more sensitive than p53-IHC, 56% of dysplasia cases that were diagnosed as negative by p53-IHC were positive (Table III). Since PHLDA1-IHC is more sensitive than p53-IHC, it is more useful as a screening diagnosis to ensure diagnostic accuracy, and when combined with the highly sensitive PHLDA1, it may be a useful auxiliary diagnostic marker. Therefore, it may be possible to include, by PHLDA1-IHC, those cases that have been determined to be negative by p53-IHC and that are difficult to determine pathologically. In addition, PHLDA1 had a high PPV, which suggests that it may be more effective, especially for dysplasia that occurred in the proximal colon (Table SIII).

However, one limitation of this study includes its small sample size. This is because we collected multiple samples (pathological sections) from a single patient. However, as far as possible, we collected different and individual tissues from that patient, including UC-CRC, dysplasia, inflammatory, and other tissues. Moreover, the histological type was as different as possible in the tissues collected from that patient, e.g., UC-CRC tissue and cells. We collected and examined dysplasia from the section that appeared to be strongly atypical as well as from the section where cell atypia was likely to be weak, the section with strong inflammation, and the section with weak inflammation. Furthermore, this study was only verified by immunostaining since no blood and stool samples were collected, which could otherwise further verify *PHLDA1* expression. A future prospective study that includes a larger number of endoscopic biopsy samples along with long-term surveillance with endoscopy is needed.

In conclusion, *PHLDA1* was believed to be involved in UC carcinogenesis, and PHLDA1-IHC has been suggested to contribute to the diagnosis of dysplasia when used in combination with p53-IHC.

## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

FO was responsible for the study conception and design, the data collection and analysis, performing the experiments, writing the manuscript and the statistical analysis. TI, MI and SO contributed to the study conception and design, data analysis, and data interpretation, and provided technical and material support. SY, AK, TM, MT, HU and YK contributed to the study conception, data interpretation, and critically revised the manuscript. FO and TI confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

The present study was conducted in accordance with the Declaration of Helsinki and its later amendments or comparable ethical standards. The study protocol was approved by the Institutional Review Board of Tokyo Medical and Dental University (approval. no. D2000-831; date, January 1, 2000 to June 30, 2025; Tokyo, Japan), and written informed consent was obtained from all the patients before enrollment.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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