**Abstract.** Background: The expression of doublecortin-like kinase 1 (DCLK1) has been investigated in cancer; however not in precancerous adenomatous polyps. Materials and Methods: Immunohistological expression of DCLK1 was evaluated in various grades of adenomas, cancerous polyps, and hyperplastic polyps in resected human tissue specimens. Results: Ninety-two specimens were positive for DCLK1 and 134 were negative. Cancerous polyps showed a high DCLK1 positivity rate compared to adenomas (68.4% vs. 36.8%; p<0.01). The rate of DCLK1 positivity was not significantly different among the three grades of adenomas (mild, moderate, and severe). DCLK1 was highly positive in advanced adenomas than low risk adenomas (49.6% vs. 29.3%; p<0.01). Conclusion: The expression of DCLK1 was found in low-grade adenomas and increased with worsening severity of dysplasia. DCLK1 expression was highly observed in advanced adenomas, which had a clinically higher malignant potential.

Doublecortin-like kinase 1 (DCLK1) has been recognized as a possible marker for intestinal cancer stem cells (CSCs). Originally, it was known as a member of doublecortin family proteins, which are microtubule-associated kinases that regulate neuronal migration (1). Recently, DCLK1 has been reported to be overexpressed in several cancers, including lung (2), esophagus (3), pancreas (4, 5), kidney (6), breast (7), and colon (8-10). Previous research has postulated that DCLK1 expression is tightly associated with cancer growth, epithelial-to-mesenchymal transition (EMT), and tumor metastasis (11-18). In addition, high expression of DCLK1 has been reported to correlate with poor prognosis in human colorectal cancer (CRC) patients (10, 19, 20).

Compared to leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5) or CD133, which are known markers for both normal and tumor stem cells in the intestine (21, 22), DCLK1 is considered a marker of tumor stem cells, although it is also expressed by normal stem cells. (12). Therefore, several promising results on the use of DCLK1 as an anticancer therapeutic target have been reported (14, 23-25). Several studies have reported on the association of colorectal polyps with LGR5 (22, 26-30) and CD133 (31-33). Takeda et al. suggested that the increase in the number of stem cells associated with LGR5 expression occurs early during colorectal tumorigenesis (26). Kazama et al. reported that CD133 expression is associated with status of differentiation and tumor size (33). Concerning DCLK1 expression in human colorectal polyps, there have been only a few reports. Gagliardi et al. (19) analyzed 18 adenomatous polyps and reported that staining score was not significantly associated with polyp location, size, morphology, architecture, degree of dysplasia, or the presence of carcinoma in situ. In contrast, Sarkar et al. (34) have reported that DCLK1 expression is stronger in both adenomas and adenocarcinomas than in normal colon mucosa, and moreover, it increases in the order of normal mucosa→adenoma→adenocarcinoma. In any case, both studies included only a small number of adenomas. Although DCLK1 is a good candidate therapeutic target, its expression in precancerous polyps or early cancerous lesions is unclear. In this study, we investigated DCLK1 expression in 226 polyps, including cancerous polyps (submucosal invasive carcinoma and carcinoma in situ), which were resected by colonoscopy. The aim was to clarify whether DCLK1 expression is correlated...
with the severity of dysplasia in colorectal adenomas. Correlations between the expression statuses of DCLK1 and prognosis of the patients were also analyzed.

Materials and Methods

Patient recruitment. A total of 226 available endoscopically-resected colorectal specimens were collected from 185 patients who underwent colonoscopy at the department of surgical oncology of the University of Tokyo our department from 1993 to 1996. All specimens were formalin-fixed and paraffin-embedded. Specimens with small size (1 mm) or not suitable for immunostaining were excluded.

Data on age, gender, survival information and location of the polyps were collected from the medical records of the patients. All specimens were examined histopathologically; adenomas were classified by the degree of dysplasia (mild, moderate, or severe), whereas cancerous polyps were classified by the depth of invasion (carcinoma in situ or submucosal invasive carcinoma), according to the Japanese Classification of Colorectal Carcinoma, 8th edition (35).

Advanced adenoma, described by Winawer et al. (36), is the most frequently used terminology to indicate adenomas with high potential for malignancy. Advanced adenoma is defined as an adenoma with at least one of the following three features: 1) the presence of villous features (more than 25%), which is defined as a tubulovillous or villous adenoma according to WHO classification; 2) size of 1 cm or more; and 3) high-grade dysplasia or submucosal invasive carcinoma. We examined the polyps using this category. This study was approved by Tokyo University Hospital Ethics Committee. Written informed consent was obtained from all participants.

DCLK1 immunostaining. The tumor specimens fixed in 10% formalin solution, embedded in paraffin blocks, were cut into 4-μM-thick sections and immunohistochemically stained as previously described (10). The sections were incubated with primary anti-DCLK1 rabbit polyclonal antibody (Ab31704, Abcam, Cambridge, MA, USA) in a 1:80 dilution overnight at 4˚C, after heat-induced antigen retrieval with an autoclave. The tissue sections were further incubated with the Dako Envision kit (Dako, Carpinteria, CA, USA), following the manufacturer’s recommendations. Reactivity was visualized in 2% 3,3’-diaminobenzidine tetrahydrochloride and 50 mM tris-buffer containing 0.3% hydrogen peroxidase.

Evaluation of DCLK1 immunostaining. Evaluation of immunostaining was performed as previously described (37). Staining intensity was categorized in four levels as follows: 0=None; 1=weak; 2=moderate; and 3=strong (Figure 1). The percentage of cells that were stained at each level was manually evaluated (0%-100%). The cumulative percentage of the stained cells was calculated and multiplied by the staining intensity (range, 0-300). The cut-off value was determined as 20 by reference to evaluation criteria of Ikemoto et al. (8). Analysis was performed independently and blindly by two observers (A.T. and H.T.) and any discrepancy was resolved by discussion. Subsequently, the correlation between DCLK1 expression and the clinicopathologic features of specimens/patients was analyzed.

Statistical analysis. The differences between the DCLK1 positive polyps and negative ones were analyzed by Chi-square test or Fisher’s exact test for categorical variables, Student’s t-test for normally distributed continuous variables, and Mann-Whitney U-test for non-normally distributed continuous variables. Correlation of overall survival (OS) between patients with DCLK1-positive polyps and patients with DCLK1-negative polyps was analyzed by log-rank test.

All analyses were performed with the JMP v13.0 (SAS Institute Inc., Cary, NC, USA). Differences with a p-value <0.05 were considered statistically significant.

Results

Patient and polyp characteristics. The median age of patients was 60 years (range, 34-90 years) and 80.6% were men. The characteristics of the polyps are shown in Table I. The size of the polyps ranged from 1 to 35 mm; 51.3% of the polyps measured ≥10 mm. The location was the right side of the colon (i.e., cecum, ascending colon, and transverse colon) in 70 specimens (31.4%); the left side of the colon (i.e., descending and sigmoid colon) in 112 specimens (49.5%); and the rectum in 44 specimens (19.5%). Based on histopathologic evaluation, 11 polyps had submucosal invasion; 27 were carcinoma in situ; 28, 126, and 17 were adenomas with severe, moderate, and mild atypia, respectively, while 17 were hyperplastic polyps.

DCLK1 expression and clinicopathologic features. Among 226 specimens, 92 were positive and 134 were negative for DCLK1 (Table II). DCLK1 positivity rate was calculated for each histopathological category, and it increased in the following order: hyperplastic polyp (17.7%); adenoma with

| Table I. Characteristics of the patients (N=185) and colorectal polyps (N=226). |
|-------------------------------|-----------------|------------------|
|                              | Patients        | Polyps           |
|                              | n               | %                |
| Gender                       |                 |                  |
| Male                         | 149             | (80.5)           |
| Female                       | 36              | (19.5)           |
| Median                       | 60              | 34-90            |
| Location                     |                 |                  |
| Right colon                  | 70              | (31.0)           |
| Left colon                   | 112             | (49.5)           |
| Rectum                       | 44              | (19.5)           |
| Size                         |                 |                  |
| <10 mm                       | 110             | (48.7)           |
| ≥10 mm                       | 116             | (51.3)           |
| Histopathology               |                 |                  |
| Hyperplastic polyp           | 17              | (7.5)            |
| Adenoma with mild atypia     | 17              | (7.5)            |
| Adenoma with moderate atypia | 126             | (55.7)           |
| Adenoma with severe atypia   | 28              | (12.4)           |
| Carcinoma in situ            | 27              | (12.0)           |
| Submucosal invasive carcinoma| 11              | (4.9)            |
mild atypia (29.4%), moderate atypia (35.7%), and severe atypia (46.4%); carcinoma in situ (66.7%); and submucosal invasive adenocarcinoma (72.7%) (Figure 2). Cancerous polyps showed a high positive rate compared with adenomas (68.4% vs. 36.8%, p<0.01). The rate of DCLK1 positivity was not significantly different among the three grades of adenomas (mild, moderate, and severe), but there was a tendency to increase with worsening severity of dysplasia (Figure 2).

Following the WHO classification (38), low-grade adenomas are those with mild and moderate dysplasia, whereas high-grade adenomas are those with severe dysplasia and carcinoma in situ. Using these definitions of severity, repeat analysis showed significant differences in DCLK1 positivity (56.4% in high-grade adenomas vs. 35.0% in low-grade adenomas, p<0.01).

**DCLK1 expression in advanced adenomas.** According to the criteria defined in Materials and Methods section, 123 of 209 specimens met the definition of advanced adenoma. DCLK1 expression was highly positive in advanced adenomas, but was rarely positive in non-advanced adenomas (49.6% vs. 29.3%, p<0.01). Based on the classification by architecture, adenomas with villous features showed higher DCLK1 positivity than other polyps but it was not statistically significant (62.5% vs. 39.0%, p=0.06). With regard to size, the rate of DCLK1 positivity was significantly higher in polyps ≥10 mm than in polyps <10 mm (53.5% vs. 27.3%, p<0.01). Similar to the results mentioned above, the group with high-grade adenomas or submucosal invasive carcinoma showed higher DCLK1 positive rate than the group with low-grade adenomas (59.1% vs. 33.1%, p<0.01) (Table II).

**OS of the patients.** The 5-year OS of patients with DCLK1 positive polyps and patients with DCLK1 negative polyps were 100% and 98.4%, respectively. No statistical significant difference was found between these two groups (p=0.38).
Similar tendencies were shown in 10-year OS (96.2% and 95.9%, respectively; \( p = 0.74 \)) and 20-year S (92.0% and 75.6%, respectively; \( p = 0.27 \)).

**Discussion**

In this study, DLCK1 expression was observed even in low-grade adenomas and increased along with the increased severity of dysplasia. Furthermore, the highest expression of DLCK1 was found in carcinomas, consistent with the sequential process of carcinogenesis. The expression of DLCK1 was significantly higher in advanced adenomas, which are adenomas with a higher risk for malignancy.

In 2006, DLCK1 was first described as a stem cell marker in adult mouse gastric and small intestinal epithelial progenitors (39). Previously, DLCK1 expression in tumor tissue has been confirmed by many reports on mice with dextran sulfate sodium-induced colitis (23), which is an APC knockout model (11, 12), and on human colorectal cancers (8, 10, 19). In a study on CRC metastasis, Gao et al. reported that DLCK1 expression was significantly increased in primary CRC and in lymphatic metastases compared with normal colorectal specimens, and moreover, DLCK1 mRNA levels in CRC were significantly correlated with lymph node metastasis and TNM stage (10). DLCK1 expression has also been strongly associated with EMT (18, 20). Although a number of genetic studies on DLCK1 have been reported, only few reports have investigated the behavior and distribution of DLCK1 expression in human CRC tissues.

For LGR5 expression, Takeda et al. (26) suggested the distribution of positive cells in a colorectal model; the location of LGR5 expression was at the base of crypts in normal epithelial cells, on the luminal surface in low-grade neoplasia, and on the bottom of the crypt and/or in invasive tumor front in high-grade neoplasia and adenocarcinoma. The presence of cancer stem cells on the tumor invasive front was suggested to correlate with metastasis and/or EMT. In a study on DLCK1 expression, Nakanishi et al. (12) observed DLCK1-positive cells at the base of the polyps in a murine model. Moreover, Ikezono et al. (8) presented a picture of DLCK1-positive cells on the invasive front of the mucosal layer in rectal neuroendocrine tumor. However, none of the studies focused on the changes in distribution of DLCK1 expression. In the present study, no statistical analysis of the distribution of DLCK1 expression in tissues was performed. Thus, whether the pattern of DLCK1 expression was similar (or not) to the previously reported distribution of LGR5 expression during each stage of
colorectal carcinogenesis remains unclear. Further studies are needed to investigate the distribution of DCLK1 expression according to the stage of colorectal carcinogenesis.

DCLK1 has also been examined as an anticancer therapeutic target. The findings with the most impact were reported by Nakanishi et al. (12), who showed that ablation of DCLK1 in murine models resulted in tumor shrinkage without damage to the normal intestine. In contrast, selective ablation of LGR5-positive cells did not lead to tumor regression and the presence of proliferative LGR5-negative cells on tumors persisted in a murine model (40). For the association of CD133 with CRC treatment, several studies have reported that CD133-positive CRC might be resistant to chemotherapy or chemoradiotherapy (41-43). Moreover, CD133-targeted oncolytic measles virus has been shown to specifically eliminate CD133-positive tumor cells (glioblastoma and hepatocellular carcinoma) in a murine model (44); however, fatal neurotoxicity was developed in this model. Based on these studies, DCLK1 seems to be the most promising target for CRC treatment, at present. Our results support the idea that therapeutic methods targeted to DCLK1 might not only affect CRC, but also cancerous and precancerous polyps. This new therapeutic approach may reduce the use of invasive methods, such as surgery and endoscopic resection, in CRC treatment.

There were several limitations to this study. The number of specimens evaluated was relatively small for this retrospective study and there might have been biases during patient recruitment. Some information about the past history of polyps and other diseases of the intestine, as well as family history, was lacking and not evaluated. In addition, the preceding studies about DCLK1 immunostaining are few and there has been no consensus on their evaluation criteria. Although the evaluation criteria adopted in this study were rather arbitrary, we hope that these would be of assistance to subsequent researchers.

**Conclusion**

The expression of DCLK1, which is a possible cancer stem cell marker, was found in low-grade adenomas and increased with worsening severity of dysplasia. It was also highly observed in advanced adenomas, which have a higher malignant potential.

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