α-Taxilin overexpression correlates with proliferation activity but not with prognosis of colorectal cancer

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Abstract. α-Taxilin is a binding partner of syntaxins, which are the central coordinators of membrane traffic. Expression of α-taxilin has been implicated in the development of human glioblastoma, hepatocellular carcinoma, and renal cell carcinoma. In this study we investigated the clinical significance of α-taxilin expression in colorectal cancer (CRC). We first analyzed 20 cases of colorectal intramucosal adenocarcinoma (IMA) with adenoma by immunohistochemical analysis and found that α-taxilin expression levels were significantly associated with Ki-67 indices in both adenoma and IMA. The patients expressed equally high levels of α-taxilin in the upper third of the intramucosal glands. These results suggested that α-taxilin expression was significantly associated with proliferation activity of colorectal tumors but that its overexpression alone could not be a marker of malignancy. We next investigated α-taxilin expression in 57 advanced CRCs in association with prognosis. Well-differentiated and/or moderately differentiated adenocarcinomas in the left-sided colon with anatomic stage II and/or III were analyzed. α-Taxilin expression levels were high on the surface of nearly all tumors but variable at the deep advancing edge. α-Taxilin levels at the advancing edge were not significantly associated with local invasiveness or prognosis. In conclusion, α-taxilin is a cell proliferation marker in colorectal epithelial neoplasms but cannot be a marker of malignancy or prognosis of CRCs.

Introduction

Colorectal cancer (CRC) is the third most common cancer and the fourth leading cause of cancer-related death worldwide (1, 2). In 2012, 1,360,600 new cases were diagnosed and 693,900 deaths were attributed to CRC (2). The carcinogenetic mechanism of CRC has been extensively investigated and intracellular signaling pathways related to cell survival, cell fate, and genome maintenance have been implicated (3).

Membrane traffic is a fundamental intracellular transport system in eukaryotic cells, and recent studies have revealed that molecules involved in membrane traffic play an important role in the initiation and progression of several types of tumor (4-6). Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) located on intracellular vesicles and their target membranes are the central coordinators of membrane traffic (7), and syntaxin family proteins are the main components of SNARE complexes. Taxilin was identified as a novel binding partner of syntaxins (8). The taxilin family consists of at least three members: α-, β-, and γ-taxilins. α-Taxilin binds to free syntaxins that are not part of a SNARE complex (9), suggesting that α-taxilin may act as a regulator of vesicular transport by affecting the assembly of SNARE complexes. In the physiological setting α-taxilin was proposed to be involved in Ca^{2+}-dependent exocytosis in neuroendocrine cells (8), and it was recently reported that α-taxilin is normally expressed in gastrointestinal epithelial cells (10). α-Taxilin is also expressed in stromal cells, such as mouse fibroblast NIH3T3 (10) and human fibrosarcoma HT1080 (11) cell lines. Overexpression of *α-taxilin* mRNA has been reported in human glioblastoma compared with normal tissues of the central nervous system (12). α-Taxilin expression has also been found to be associated with

proliferative activity and dedifferentiation of hepatocellular carcinoma (HCC) (13) and local invasiveness and poor prognosis of renal cell carcinoma (RCC) (14).

In the present study, we analyzed α-taxilin expression in human colorectal tumors and explored the associations between α-taxilin overexpression and prognosis of CRC. This is the first study to investigate the clinical significance of α-taxilin in human CRC.

Materials and methods

Tumor Samples, Pathological Diagnosis, and Staging. CRCs that were endoscopically or surgically resected at the Dokkyo Medical University Hospital (DMUH) and the International University of Health and Welfare, Shioya Hospital (IUHWSH) were analyzed. Clinicopathologic classification was based on the World Health Organization classification of colorectal tumors and stage grouping was according to tumor, node, metastases (TNM) staging of the American Joint Committee on Cancer (AJCC) Colon and Rectum Cancer Staging (15, 16).

Colorectal intramucosal adenocarcinomas (IMAs; pTis defined by AJCC) with adenoma components were selected from all CRCs resected at IUHWSH from 2013 to 2015 and at DMUH from 2012 to 2014. A total of 20 IMAs, pathologically diagnosed as well-differentiated and/or moderately differentiated adenocarcinoma, were analyzed. For pathological analysis, intramucosal glands were divided into three anatomical components: upper third (UT), middle third (MT), and lower third (LT) (Fig. 1A). Next, among all CRCs surgically resected at DMUH and IUHWSH from 2009 to 2011, histologically proven well-differentiated and/or moderately differentiated adenocarcinomas in the left-sided colon with anatomic stage II and/or III were selected.

All CRCs included in this study were diagnosed clinically and pathologically as primary tumors and confirmed to be advanced cancers that invaded the muscularis propria or more (pT2 to 4). We aimed to study the pure effect of α-taxilin expression on prognosis by normalizing other possible prognostic factors, such as tumor histology, differentiation grade, location, and initial anatomic stage (17). Patients with complete medical records were included in the survival analyses, but individuals with invasive cancers originating from other sites were excluded. As a result, a total of 57 cases were subjected to prognostic analyses. This study protocol was approved by the ethical review boards of the participating hospitals (IUHWSH, FK-94; DMUH, 26067).

Immunohistochemistry. Tumor specimens were fixed in 10% neutral-buffered formalin for 48 h, embedded in paraffin, and then cut into 4-μm sections. Antigen retrieval was performed in 10 mM citrate buffer (pH 6.0) using microwave irradiation (400 W) at 95°C for 40 min. After quenching endogenous peroxidase activity, sections were incubated with primary antibody detecting α-taxilin (1:1,000) or Ki-67 (1:50, clone: Mib-1; Dako, Glostrup, Denmark) for 60 min at room temperature. Characterization of the anti–α-taxilin antibody was described in previous studies (10, 18). Intensity of α-taxilin staining was classified into four categories: level 3, comparable to that of proliferating cells in the lower crypt, follicular dendritic cells, and immunoblasts: level 2, comparable to that of ganglion cells and follicular B-lymphocytes: level 1, between level 0 and 2: level 0, no staining (Fig. 1B and 1C). α-Taxilin expression of levels 2 and 3 was regarded as overexpression. Ki-67 indices were calculated as the percentage of Ki-67–positive cells among 500-1,000 cells in the areas with the strongest nuclear labeling.

Statistics. Comparisons between two sets of data were performed by non-paired/paired two-tailed Student's *t* test. Correlation between α-taxilin expression levels and Ki-67 indices were analyzed by Spearman's rank correlation analysis. Specific parameters between two patient cohorts were compared using the χ^2 test with/without Yates' correction or by Fisher's exact test. Age was compared using the Mann–Whitney *U* test, and survival curves were analyzed using the Kaplan–Meier method and log-rank tests. A p value < 0.05 was considered significant. Statistical analyses were performed using IBM SPSS Statistics 23 (IBM, Armonk, NY, USA).

Results

α-Taxilin Expression in IMA and Adenoma. A total of 20 cases of IMA with adenoma were analyzed (Fig. 2A). The proliferative zone was in the LT in the normal colonic crypt, whereas it shifted to UT in adenoma and spread from the UT downward in IMA (Fig. 2B). α-Taxilin expression paralleled Ki-67 expression (Fig. 2B and 2C). In the normal mucosa, expression of both α-taxilin and Ki-67 was strongest in LT followed by MT, and weakest in UT of the crypt (Fig. 3A). In contrast, for glands in adenoma and IMA, expression of both α-taxilin and Ki-67 was strongest in UT followed by MT, and weakest in LT (Fig. 3A). α-Taxilin levels were similarly high in UT of glands of adenoma and IMA (2.813 ± 0.527 and 2.875 ± 0.484, respectively), although Ki-67 indices were significantly higher in IMA than in adenoma (88.46 ± 9.343 and 63.35 ± 17.2, respectively: $p < 0.01$) (Fig. 3A).

Coordinate points consisting of α-taxilin level in the x-axis and Ki-67 index in the

y-axis in respective anatomical components (LT, MT, and UT) of intramucosal neoplastic glands were plotted in a scattergram (Fig. 3B). α-Taxilin level and Ki-67 index showed a significant positive correlation in both adenoma and IMA.

α-Taxilin Expression in CRC and Prognosis. We next investigated α-taxilin expression in histologically proven well-differentiated and/or moderately differentiated adenocarcinoma in the left-sided colon with anatomic stage II and/or III and examined its association with local invasiveness and prognosis. α-Taxilin was strongly expressed at the cancer surface of nearly all CRCs: level 1 in 6 cases, level 2 in 15 cases, level 3 in 35 cases, not determined in one case (Fig. 4A and 4C). In contrast, α-taxilin expression levels at the cancer deep advancing edge in the colorectal wall were more variable: level 0 in 4 cases, level 1 in 12 cases, level 2 in 25 cases, and level 3 in 16 cases (Fig. 4B and 4D). Sixteen of 57 cases (28.1%) showed low expression of α-taxilin. On average, there was a significant difference in α-taxilin expression levels between the surface area and the advancing edge (2.52 ± 0.09 and 1.91 ± 0.12, respectively; $p < 0.01$) in the present CRC cohorts.

Malignant tumor is characterized by destructive downward growth and metastasis. We therefore investigated associations of α-taxilin expression in cancer cells at the advancing edge with local invasiveness and prognosis. α-Taxilin overexpression (levels 2 and 3) was not associated with any of the clinical parameters tested, including depth of tumor (pT), venous invasion, lymphatic permeation, nodal metastasis, and clinical stage (Table 1). In the survival analyses, CRCs with α-taxilin overexpression showed a trend for worse 5-year recurrence-free survival than those without α-taxilin overexpression, but the difference did not reach statistical significance (Fig. 5A).

Advanced CRCs with and without α-taxilin overexpression demonstrated similar 5-year overall survival (Fig. 5B). These results suggest that the α-taxilin expression level may not affect prognosis of CRCs.

Discussion

It has been reported that α-taxilin is related to cell proliferation of neuroepithelial cells and malignancies of epithelial origin, such as HCC and RCC (8, 13, 14). Recently, Horii et al. reported α-taxilin expression status in the normal gastrointestinal tract in mice (10). They demonstrated that α-taxilin was expressed in the majority of the gastrointestinal tract and prominently present in the cytoplasm of epithelial cells expressing Ki-67 in C57BL/6 mice. Although some epithelial cells expressed α-taxilin without Ki-67 expression, the majority of α-taxilin–positive/Ki-67–negative epithelial cells were observed in the vicinity of α-taxilin/Ki-67 double-positive cells, and the authors reasoned that α-taxilin remained in these cells following division and subsequent loss of Ki-67 expression. They also reported that treatment with dibenzazepine, a γ-secretase inhibitor that inhibits cell proliferation in the stomach, resulted in a decrease in the number of Ki-67–positive cells and α-taxilin–positive cells in the lower part of the gastric glands, suggesting that expression of α-taxilin was dependent on cell proliferation. In the present study, we investigated α-taxilin expression status in human colorectal tumors for the first time. Assuming that α-taxilin might be a marker of CRC, we first studied its expression focusing on the pathological sequence of colorectal carcinogenesis. In normal mucosa, the proliferation zone of the colonic crypt is located in the lower third of the crypt, where stem cells are present.

However, the proliferation zone represented by high Ki-67 indices shifted to the upper third of the crypt in adenoma and was found to extend downward from the UT in IMA. These observations corresponded well with one of the two models explaining colon carcinogenesis: the top-down model. This model proposes that transformation is initiated in a fully differentiated cell in which adenomatous polyposis coli is highly expressed and β-catenin is downregulated (19). The fully differentiated villus cell proliferates and replaces the normal mucosa from the top down. In contrast, the bottom-up model proposes that transformation is initiated in a stem cell at the base of the crypt, which proliferates and replaces the normal mucosa with transformed cells from the bottom up. In the present study, α-taxilin levels were significantly associated with proliferation activity in both adenoma and IMA. We observed that α-taxilin was similarly upregulated in the upper third of the neoplastic glands in both adenoma and IMA. These data suggested that α-taxilin expression status might be similar between adenoma and adenocarcinoma, and that high α-taxilin levels could not be a marker of malignancy in the large intestine.

We next investigated α-taxilin expression in histologically proven well-differentiated and/or moderately differentiated adenocarcinoma in the left-sided colon with anatomic stage II and/or III in order to investigate its prognostic significance in CRC. Since α-taxilin expression levels were similarly high in the surface area of adenoma and adenocarcinoma, we reasoned that α-taxilin expression on the surface could not be a marker of malignancy or prognosis in CRCs. However, the malignant potential of cancer would be determined by proliferation activity, destructive downward growth, and metastasis, suggesting that malignant potential may be determined by the invasiveness and proliferation activity of cancer cells at the deep advancing edge. We therefore

investigated associations of α-taxilin expression at the advancing edge with local invasiveness (pT and vessel invasion) and prognosis; however, α-taxilin expression levels were not associated with local invasiveness and also did not affect 5-year recurrence-free survival or overall survival of patients with advanced CRCs.

Previously, Ohtomo et al. reported that α-taxilin upregulation was significantly associated with proliferative activity, less-differentiated histological grade, positivity of vascular invasion, and/or intrahepatic metastasis of HCC (13). In addition, Mashidori et al. reported that α-taxilin upregulation was significantly associated with depth of tumor (pT), vessel invasion, and unfavorable overall and disease-free survival of patients with RCC (14). In the present study, α-taxilin expression levels were associated with proliferation activity but its overexpression did not significantly affect prognosis. There are some possible explanations for this discrepancy. First, it is well known that a majority of CRCs develop via the adenoma-carcinoma sequence. Because α-taxilin levels in adenoma cells are already as high as those in adenocarcinoma cells, upregulation of α-taxilin alone cannot be a marker of malignant potential of colorectal neoplasms. In contrast, except for a few cases, no such common precancerous lesion has been reported for HCC and RCC. The majority of both tumors may develop *de novo*, and therefore α-taxilin upregulation could be a marker of malignancy. Second, we selected a relatively homogenous group of CRCs to investigate only the effect of α-taxilin expression on prognosis. We enrolled only cases of histologically proven differentiated adenocarcinoma in the left-sided colon with anatomic stage II and/or III that underwent surgical resection. However, this selection might have resulted in selection bias because α-taxilin levels might have affected pT and nodal/distant metastasis. Analysis of all consecutive CRC cases in our regional cohort during the

study period might have resulted in a different conclusion. This is definitely a limitation to this trial, in part because patients with stage I disease would undergo endoscopic treatment and those with stage IV disease may not undergo surgical resection. Third, histological structure differs greatly between CRC and HCC/RCC; invasive CRCs are relatively stroma-rich tumors whereas both HCCs and RCCs are deficient in fibrous stroma. In the present study, as well as in previous studies, α-taxilin expression was also noted in the stromal cells such as fibroblasts. Previous studies suggested fibroblasts as the cellular origin of matrix metalloproteinase (MMP)-9 in CRCs (20, 21). Increased MMP-2 and MMP-9 expression has been associated with worse outcome of CRC (22-24). The fact that α-taxilin preferentially binds to free syntaxins and thereby prevents formation of the SNARE complex suggests that α-taxilin might act as a negative regulator of t-SNARE formation, leading to impaired intracellular vesicular transport (8, 9). SNAREs have been reported to be involved in secretion of MMP-2 and MMP-9 (25), therefore α-taxilin expression in fibroblasts might cause decreased MMP-9 secretion, leading to a favorable prognosis of CRCs. α-Taxilin expression in both cancer cells and stromal cells may offset the prognostic effect in stroma-rich CRCs.

In conclusion, this is the first report of α-taxilin expression in human colorectal tumors. In both adenoma and adenocarcinoma, α-taxilin was significantly associated with cell proliferation activity. However, α-taxilin expression levels were not significantly associated with local invasiveness or recurrence-free/overall survival of advanced CRCs. Taken together, our findings indicate that α-taxilin is a cell proliferation marker in colorectal epithelial neoplasms, but cannot be a marker of malignancy, and its expression does not affect prognosis of CRC.

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Figure 1. (A) Three anatomical components of normal colonic crypts: upper third, middle third, and lower third. (B) α-Taxilin expression in the colonic crypts and ganglion cells. Level 3, comparable to expression of proliferating cells in the lower crypt (arrows); level 2, comparable to expression of ganglion cells (arrowheads). (C) α-Taxilin expression in the lymph follicles. Level 3, comparable to expression of follicular dendritic cells (arrowheads) and immunoblasts (arrows); level 2, comparable to expression of follicular B lymphocytes (in the circle).

Figure 2. (A) Representative histological structure of IMA with adenoma (H&E ×2). (B) Ki-67 expression status (×2). (C), α-Taxilin expression status (×2). IMA, intramucosal adenocarcinoma.

Figure 3. (A) α-Taxilin expression levels and Ki-67 labeling indices in the respective anatomical units (lower, middle, and upper thirds) of the normal crypt, adenoma, and IMA. (B) Scattergrams displaying correlations between α-taxilin expression level and Ki-67 labeling index in three anatomical units of adenoma and IMA. U, upper third; M, middle third; L, lower third; IMA, intramucosal adenocarcinoma.

Figure 4. Representative α-taxilin expression status at the surface and at the deep advancing edge of advanced CRC (×4). Level 3 staining at the surface (A) and at the advancing edge (B) in the sigmoid colon cancer of an 80-year-old male patient. Arrows indicate ganglion cells. Level 3 staining at the surface area (C) and level 1 staining at the advancing edge (D) in rectal cancer of a 78-year-old female patient. Arrows indicate ganglion cells. CRC, colorectal cancer.

Figure 5. Survival analyses of histologically proven well-differentiated and/or moderately differentiated adenocarcinoma in the left-sided colon with anatomic stage II and/or III. (A) Five-year recurrence-free survival in the presence (n = 41; solid line) or absence (n = 16; dotted line) of α-taxilin overexpression at the deep advancing edge. (B) Five-year overall survival in the presence (n = 41; solid line) or absence (n = 16; dotted line) of α-taxilin overexpression at the deep advancing edge.