Down-Regulation of PIK3CG, a Catalytic Subunit of Phosphatidylinositol 3-OH Kinase, by CpG Hypermethylation in Human Colorectal Carcinoma

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ABSTRACT

**Purpose:** Premature death associated with colorectal adenocarcinoma occurs in PIK3CG−/− mice and overexpression of PIK3CG in colon cancer cells suppresses cell proliferation. We examined expression levels of PIK3CG, a catalytic subunit of phosphatidylinositol 3-OH kinase (PI3K), in colon cancer cells to investigate the hypothesis that PIK3CG might contribute to the growth and progression of colorectal cancers.

**Experimental Design:** The effects of LY294002, a PI3K inhibitor, on cell growth were examined to elucidate the role of the PI3K-Akt/protein kinase B (PKB) pathway in colon cancer. We used reverse transcription-PCR, Western blotting, and immunohistochemical analyses to examine PIK3CG mRNA and protein expression levels in colon cancer cells and primary colorectal cancers. To clarify the mechanism responsible for the silencing of this gene in colon cancers, we performed methylation-sensitive PCR analysis of DNA digested with HpaII and MspI and analyzed PIK3CG expression in DLD-1 and LoVo cells treated with the demethylating agent 5-aza-2′-deoxycytidine (5-Aza).

**Results:** LY294002 suppressed growth and decreased expression of Akt (Ser473) expression in cancer cells. Three (60%) of 5 colon cancer cell lines did not express PIK3CG, but hypermethylation at CpG sites of the promoter regions of this gene was detected. However, 5-Aza-treated DLD-1 and LoVo cells did express PIK3CG. Reduction of PIK3CG expression was detected immunohistochemically in 85% of human colorectal cancers and was closely associated with invasion, metastasis, and poor differentiation. Down-regulation of PIK3CG expression and hypermethylation of promoter regions were also detected in primary colon cancers.

**Conclusions:** Our findings suggest that the silencing of the PIK3CG gene plays an important role in inhibiting the PI3K-Akt/PKB signaling system responsible for tumorigenesis and the progression of colorectal cancers.

INTRODUCTION

PI3K is essential for regulation of various intracellular signals involving lipid second messengers. Growth factors, such as epidermal and platelet-derived growth factors activate receptors that recruit PI3K to cell membranes, leading subsequently to the formation of phosphatidylinositides. Among the various intracellular signaling pathways, the PI3K-Akt/PKB pathway is believed to play a critical role in cell survival, and its activation is linked to tumorigenesis. Up-regulation of Akt and its upstream regulator PI3K has been observed in various human malignancies, and the negative regulator of this pathway PTEN/MMAC is a tumor suppressor. In a previous study, we demonstrated that phosphorylation of Akt occurred frequently in colorectal carcinomas and was required for the suppression of cell growth and the blockade of apoptosis, whereas no alterations of the PTEN/MMAC gene have been detected in human colorectal cancers. Thus, the details of the mechanism that regulates the PI3K-Akt/PKB pathway in colorectal cancers remain unclear.

Four isoforms of mammalian type 1 PI3K (p110α, β, γ, and δ) interact with the p85 family of regulatory subunits and are divided into two groups (types 1A and 1B), according to their regulatory system. The type 1A PI3Ks (p110α, β, γ, and δ) are controlled by tyrosine-phosphorylated proteins, whereas the type 1B PI3K p110γ (PIK3CG) is activated by interaction with G-protein-coupled receptors. Therefore, the function of PIK3CG is thought to differ from the functions of the type 1A PI3Ks. Previous studies demonstrated that the homozygous deletion of the PIK3CG gene did not influence mouse embryogenesis or development, although it did cause abnormalities in neutrophil migration and T-cell function. Interestingly,
homozygous PIK3CG−/− mice have shorter life spans than their heterozygous littermates (11). Although young (<8 weeks old) mice showed no abnormalities in appearance, size or body weight, the incidence of premature death associated with colorectal adenocarcinoma may be higher in older PIK3CG−/− mice than in their heterozygous littermates. Furthermore, overexpression of PIK3CG in colorectal cancer cells suppressed cell proliferation, which suggests that loss of function of this protein may contribute to colorectal tumorigenesis (11).

In the present study, we examined the PIK3CG expression levels of colon cancer cells to explore the hypothesis that the reduction of PIK3CG expression might contribute to the growth and progression of human colon cancers. Because reduction of PIK3CG expression in colon cancer cell lines has been observed, we analyzed the mechanisms that regulate PI3KCG expression, including chromosomal deletion of the locus and CpG hypermethylation of the promoter region of this gene. Moreover, we demonstrated that reduced PI3KCG expression occurred frequently in primary colorectal carcinomas, and correlations with clinicopathological features and p53 status of the tumors were also analyzed (12).

**MATERIALS AND METHODS**

**Cell Culture and Tissue Samples.** Five colon carcinoma cell lines (DLD-1, LoVo, Colo205, HCT-15, and SW480) were used in this study. The cells were routinely maintained in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 1 mM L-glutamine, 10% FBS (Life Technologies, Inc.), and 100 units/ml penicillin G. Cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO2.

A total of 68 colorectal cancers removed at Yamagata University Hospital (Yamagata, Japan) were used for immunohistochemistry. Informed consent was obtained from all of the patients before surgery. The clinicopathological findings are summarized in Table 1. There were 43 men and 25 women, with an average age of 66.4 years (range, 38–83 years) and an average size of tumors of 43.2 mm (range, 8–110 mm) in greatest dimension. Formalin-fixed and paraffin-embedded tissue samples were used for histological diagnosis and immunohistochemistry. Histological classification and clinicopathological staging were performed according to the general rules for clinical and pathological studies on cancer of the colon, rectum, and anus (13). With regard to the depth of carcinoma invasion, we divided all of the cases into three groups: (a) intramucosal carcinoma (m); (b) carcinoma with limited invasion to submucosal region (sm) and muscularis propria (mp); and (c) carcinoma invading to serosa (ss+se) or adventitia (a1+a2). Additionally, a total of 11 fresh and frozen tumor specimens and corresponding normal colon mucosa were also obtained along with the surgically resected colorectal cancers for RT-PCR and methylation-sensitive PCR analyses. Tissues were immediately frozen in liquid nitrogen and stored until use. This study was carried out in accordance with the ethics code for Human Experimentation, Yamagata University School of Medicine.

**WST-1 Cell Proliferation Assay.** Cell growth was determined using Premix WST-1 Cell Proliferation Assay System (Takara Bio-chemicals Co., Tokyo, Japan) as described elsewhere (14). Briefly, 1.0 × 10^5 cells (100 µl volume/well), maintained in low-nutrient and phenol red-free RPMI 1640 (Life Technologies, Inc.), were inoculated into 96-well microtiter plates. The PI3K inhibitor LY294002 (Sigma, St. Louis, MO; 0–50 µM) dissolved in DMSO was added to triplicate wells and cultured in the presence (10%) or absence of FBS at 37°C for 24 h (15). After treatment, 10 µl of Premix WST-1 was added to each microculture well, and plates were incubated for 60 min at 37°C. Absorbance at 450 nm was measured with a microplate reader. The final concentration of DMSO did not affect cell survival or protein phosphorylation in our study.

**Western Blotting.** Cells were lysed in a buffer containing 50 mM Tris-HCl (pH 7.4), 125 mM NaCl, 0.1% Triton X-100 and 5 mM EDTA containing both 1% protease inhibitor (Sigma) and 1% phosphatase inhibitor cocktail II (Sigma). Protein was extracted from cells treated with 20 µM of LY294002 and each 40 µg of protein was separated by SDS-PAGE followed by electrotransfer onto a Sequi-Blot polyvinylidene fluoride membrane (Bio-Rad, Richmond, CA). For detection of PIK3CG from the cells with or without 5-Aza demethylating agent, immunoprecipitation using protein G agarose (Life Technologies, Inc.) was performed as described elsewhere (9). Phosphorylation-specific rabbit polyclonal antibody against Akt (Ser473) and mouse monoclonal antibody for PIK3CG were purchased from New England Biolabs (Beverly, MA) and Santa Cruz Biotechnology Inc. (Santa Cruz, CA), respectively. Horseradish peroxidase-conjugated goat anti-rabbit/mouse IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) was used as a secondary antibody for enhanced chemiluminescence (NELife Science Products Inc., Boston, MA). The levels of phosphorylated Akt (Ser473) expression were measured by NIH Image software.

**Mapping of Microsatellite Markers on Chromosome 7q22.1-q31.33.** To evaluate the retention or homozygous deletion of the PIK3CG locus, we amplified microsatellite markers on chromosome 7q22.1-q31.33. PCR primers used in this study were as follows: D7S2453 (forward, 5′-CTTGACCCCAG-GAAATG-3′; reverse, 5′-GTAAACTG ACCCTATGACCAAG-3′); D7S501 (forward, 5′-ACCGTTTGATGGCAGAG-3′; reverse, 5′-ATTCTTACACGGCAGACTGCT-3′); and D7S2420 (forward, 5′-CTGTATGGGAGG CAAACTA-3′; reverse, 5′-AATAATGACTGAGGCTCAAAACA-3′). Genomic DNA from each colon cancer cell or primary colon cancer as well as adjacent normal colon mucosa was extracted and PCR was carried out as reported previously (16–18). Briefly, each 15-µl reaction mixture, containing 10 ng of DNA, 6.7 mM Tris-HCl (pH 9.0), 6.7 mM EDTA, 6.7 mM MgCl2, each 0.33 mM primers, 1.5 mM dNTPs, and 0.75 units of recombinant TaqDNA polymerase (Life Technologies, Inc.) was amplified for 40 cycles with following regimen: denaturation at 94°C for 30 s; annealing at 55°C for 30 s; and extension at 72°C for 30 s. The resulting amplification products of cancer cell lines were analyzed by 3% agarose gel electrophoresis with ethidium bromide and were examined under UV light.

**RT-PCR Analysis of the PIK3CG Gene.** Total RNAs from each colon cancer cell line (1.0 × 10^5), primary colon cancers, and the corresponding normal mucosa were isolated by using RNeasy Mini Kit (Qiagen, Hilden, Germany) and RT-PCR analysis was performed with OneStep RT-PCR assay kit (Qiagen). Primer sets used for RT-PCR amplification were as follows: PIK3CG mRNA-forward, 5′-AGAGTCCATAT-
GATCCCTGG-3′; and PIK3CG mRNA-reverse, 5′-AATCTC-

TCCACTGCTGCCTG-3′. As a control, the levels of HPRT expression was also analyzed (forward, 5′-CGGGGACATA-

AAAGTTAT-3′; reverse, 5′-CCACTTTTGCG TGATGA-

CAC-3′. Each 25-μl reaction mixture containing 10 ng of total RNA, 1 μM of the primer pair, and 0.75 units of reverse transcriptase and TaqDNA polymerase was amplified for 30 cycles with the following regimen: reverse transcription at 50°C for 30 min; denaturation at 94°C for 30 s; annealing at 58°C for 30 s, and extension at 72°C for 1 min. RT-PCR products underwent electrophoresis in 2% agarose gel. Expression of PIK3CG was measured by densitogram (ATTO, Tokyo, Japan).

**Analysis of Methylation Status of Promotor Region of the PIK3CG Gene.** Two cell lines, DLD-1 and LoVo, were treated with 10 μM 5-Aza (Sigma) demethylating drug for 30 days (17). Genomic DNA samples of each colon cancer cell line were digested with restriction endonucleases in 100-μl volumes of restriction endonuclease buffer containing 5 μg of DNA from each digest was analyzed by PCR using the PIK3CG-pro primer sets (forward, 5′-TCTAGCCAGAAGGAGTGC-3′; reverse, 5′-

TCTTTATAGTTAGC CAGTG-3′) designed to amplify −723 to +26 of the PIK3CG gene. PCR was performed for one cycle of 95°C for 9 min followed by 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min, followed by one cycle of 72°C for 10 min using TaqDNA polymerase (Life Technologies, Inc.). The buffer contained 1.5 mM MgCl₂, 10 mM Tris-

HCl, 50 mM KCl, and 20 μM each dNTPs. The resulting amplification products were then analyzed by 2% agarose gel electrophoresis with ethidium bromide and examined under UV light.

**Immunohistochemical Analyses.** We confirmed that the PIK3CG mouse monoclonal antibody used in Western blotting was useful for immunohistochemistry. Additionally mouse monoclonal antibodies against Ki-67 (Immunootech, Marseille, France) and p53 (Novocastra Laboratories Ltd., Newcastle, United Kingdom) were also analyzed. An avidin-biotin complex immunoperoxidase technique was performed as described elsewhere (19). Briefly, deparaffinized tissue sections were immersed in methanol containing 0.03% hydrogen peroxide for 30 min to block endogenous peroxide activity after autoclave pretreatment in citrate buffer to retrieve the antigenicity. After incubation with normal horse serum for 30 min to block the nonspecific antibody binding sites, the sections were treated consecutively at room temperature with anti-PIK3CG (diluted 1:100), anti-Ki-67 (MIB-1; diluted 1:200), and anti-p53 (diluted 1:200) antibodies for 90 min. Subsequently, sections were biotinylated by antimouse IgG horse serum for 30 min and with avidin DH-biotinylated horseradish peroxide complex (Histofine kit, Nichirei, Tokyo, Japan) for 30 min. Peroxide staining was performed for 2–5 min using a solution of 3,3′-diamino-benzidine tetrahydrochloride in 50 mM Tris-HCl (pH 7.5) containing 0.001% hydrogen peroxide. The sections were counterstained with 0.1% hematoxylin. Immunoreactivity of anti-PIK3CG antibody was graded according to the number of stained cells and the staining intensity in individual cells as follows: negative, almost no positive cells; low, 5–50% of tumor

**RESULTS**

**The Effects of PI3K Inhibitor LY294002 and Expression of PIK3CG in Colon Cancer Cells.** First, we examined the effects of LY294002 on the growth of various colon cancer cell lines to ascertain the role of PI3K in colon cancer cell growth. LY294002 reduced the WST-1 absorbance of DLD-1, LoVo, and SW480 colon carcinoma cell lines. Cells were cultured in complete RPMI 1640 containing 0–50 μM LY2940002 for 24 h. WST-1 absorbance (450 nm) in the presence and absence of 10% FBS are illustrated. B, expression of phosphorylated Akt (Ser⁴⁷³) in colon cancer cells. When cells were treated with 20 μM LY294002, the levels of phosphorylated Akt was decreased compared with untreated cells (U), with the mean decreased Akt phosphorylation levels 45, 94, and 79% in DLD-1, LoVo, and SW480 cells, respectively.
line decreased in proportion to the magnitude of growth inhibition [DLD-1 (45%), LoVo (94%), SW480 (79%); Fig. 1B]. Growth of each Colo205 and HCT-15 cell line was also suppressed by treatment with LY294002, with decreased expression of phosphorylated Akt protein (data not shown).

To investigate allelic loss of the PIK3CG locus in this series of colon cancer cells, we performed genomic DNA PCR analysis of the microsatellite loci (chromosome 7q22.1-q31.33) and promoter region of this gene. No colon cancer cell lines showed homozygous deletion of this locus (Fig. 2A). Next, we carried out RT-PCR analysis and confirmed expression of PIK3CG in normal colonic mucosa. In cancer cell lines, no expression of PIK3CG mRNA was detected in DLD-1, LoVo, or SW480 colon cancer cells, but slight expression was observed in Colo205 and HCT-15 cells. However, we could not detect expression of PIK3CG in these cell lines by immunoblotting. Expression of HPRT is shown as a control.

Hypermethylation of the Promoter Region of the PIK3CG Gene in Colon Cancer Cells. The methylation status of four CCGG residues in the PIK3CG gene promoter was studied using PCR bridging of this region before and after DNA digestion with the isoschizomers MspI and HpaII. PCR bridging of the promoter region containing CpG islands after DNA digestion with MspI failed to amplify this region, and the highly methylated CCGG sites were resistant to HpaII digestion. Therefore, the HpaII-resistant PIK3CG promoter DNA was found to be hypermethylated (17). The promoter regions of the PIK3CG genes of this series of colon cancer cells resisted digestion with HpaII but were sensitive to digestion by MspI (Figs. 2C and 3A). However, the status of promoter region of the PIK3CG gene in Colo205 and HCT-15 cells was considered to be partially hypermethylated.

Fig. 2 Mapping of microsatellite markers on chromosome 7q22.1–31.33 and suppression of PIK3CG in colon cancer cells. A, a scheme of genomic structure at the PIK3CG gene locus (7q22.1-q31.33) in colon cancer cells (left). To detect DNA methylation at the promoter region of the PIK3CG gene, PCR primer sets (PIK3CG-pro: Forward and PIK3CG-pro: Reverse) were designed to amplify from −723 bp to +26 bp. There was no homozygous deletion at this locus in human colon cancer cell lines (right). B, expression of PIK3CG by RT-PCR analysis. No expression was detected in DLD-1, LoVo, or SW480 colon cancer cell lines, but slight expression was observed in Colo205 and HCT-15 cells. However, we could not detect expression of PIK3CG in these cell lines by immunoblotting. Expression of HPRT is shown as a control. C, DNA hypermethylation at the promoter region of the PIK3CG gene in Colo205, HCT-15, and SW480 colon cancer cells. DNA methylation was observed in these colon cancer cell lines (Colo205, HCT-15, SW480) by PCR using methylation-sensitive HpaII restriction enzyme, as well as DLD-1 and LoVo cells (Fig. 3A). However, the status of the promoter region of the PIK3CG gene of Colo205 and HCT-15 cells was considered to be partially hypermethylated.

Fig. 3 Expression of PIK3CG in DLD-1 and LoVo cells after treatment with 5-Aza demethylating agent. A, methylation-sensitive PCR in DLD-1 and LoVo cells. Although both DLD-1 and LoVo cells exhibited hypermethylation of the promoter region of the PIK3CG gene, when cells were treated with 5-Aza, demethylation at this region occurred completely in the DLD-1 cells but incompletely in the LoVo cells. U, undigested; M, MspI-digested DNA; H, HpaII-digested DNA. B, induction of transcription of PIK3CG by RT-PCR in both cells treated with 5-Aza for 30 days. C, Western blotting of PIK3CG in DLD-1 and LoVo cells. Expression of PIK3CG was confirmed in both cells after 5-Aza treatment by immunoprecipitation.
Next, DLD-1 and LoVo cells were treated with 10 μM 5-Aza for 30 days to determine the effect of this demethylating agent on the regulation of PIK3CG expression. During treatment, the cell proliferative ability decreased, and a small number of the cultured cells died. Altered sensitivity to HpaII digestion was detected in DLD-1 cells by methylation-sensitive PCR analysis, and very low levels of PCR products were detected in this region of LoVo cells (Fig. 3A). Both DLD-1 and LoVo cells treated with 5-Aza expressed PIK3CG mRNA and protein (Fig. 3, B and C).

**Immunohistochemical Analysis of PIK3CG Expression in Colorectal Cancer.** PIK3CG was detected mainly in the cell membranes of nonneoplastic normal colonic mucosa and tubular adenomas, but expression was weak in the cytoplasm of epithelial cells and tubular adenomas (Fig. 4A). No significant expression was detected in fibroblasts, smooth muscle cells, or endothelial cells, although significant expression was found in some inflammatory cells. Of a total of 68 carcinomas examined, only 10 (15%) showed high PIK3CG expression levels (Fig. 4B), whereas reduced expression of PIK3CG was detected in the other 58 (85%) cases, of which 39 (57%) and 19 (28%) showed low and no expression, respectively (Fig. 4, C and D).

The association between reduced PIK3CG expression and the clinicopathological findings was also analyzed, and the results are shown in Table 1. Reduced PIK3CG expression was detected more frequently in poorly differentiated than in well- and moderately differentiated adenocarcinomas ($P < 0.02$). In advanced cancers demonstrating massive invasion of the muscularis propria, the incidence of reduced PIK3CG expression was higher than that in intramusosal carcinomas ($P < 0.001$). Furthermore, a reduction of PIK3CG expression was closely associated with carcinoma progression, such as invasion of lymphatic vessels ($P < 0.002$) and metastasis to the lymph nodes ($P < 0.02$). High PIK3CG expression levels were closely related to noninvasive cancers (stage 0), whereas PIK3CG expression was markedly reduced in advanced-stage cancers ($P < 0.001$). However, there was no significant correlation between PIK3CG expression and the Ki-67 (MIB-1) labeling indices or p53 status of tumors.

**Reduction of PIK3CG by CpG Hypermethylation in Primary Colon Cancer.** The expression levels of PIK3CG in primary human colorectal cancer tissues and the corresponding adjacent normal mucosae were compared. The PIK3CG expression level was lower in 6 (55%) of the 11 colon cancer tissues than in their adjacent normal mucosae (Fig. 5A). Immunohistochemical analysis showed that each tumor with a low PIK3CG level had a low PIK3CG expression level. Although no chromosome 7q deletion was detected (data not shown), the PIK3CG promoter regions of 5 (45%) of the 11 colon cancers were considered to be partially hypermethylated, relatively resistant to digestion by HpaII but sensitive to digestion by MspI (Fig. 5B).

**DISCUSSION**

Lipid second messengers transduced by PI3K function in various intracellular signaling pathways (21, 22). Interest in PI3K has increased with evidence that the transforming abilities of several oncoproteins correlate with PI3K expression levels and elevated in vivo levels of specific lipid products of this enzyme (23). In the current study, we demonstrated that LY294002 inhibited the growth of several colon cancer cell
lines and reduced their phosphorylated Akt expression levels. Although the sensitivities of the cell lines to LY294002 varied, the PI3K-Akt/PKB pathway can be regarded as an important signaling mechanism for the growth and maintenance of colon carcinoma cells, as we reported previously (5, 14).

Although PIK3CG expression was observed in nonneoplastic colonic mucosa by immunohistochemistry and RT-PCR-analysis, three (60%) of the five colon carcinoma cell lines used in this study did not express this PI3K catalytic subunit. The results of the subsequent immunohistochemical examination, RT-PCR assay and methylation-sensitive PCR analysis of primary colorectal adenocarcinomas demonstrated that reduction of PIK3CG expression by silencing the PIK3CG promoter region may be one of the pivotal events in colonic tumorigenesis. To the best of our knowledge, this is the first study to show frequent reduction of PIK3CG and a mechanism that silences the PIK3CG gene in colon cancer cells. Furthermore, 5-Aza treatment of both DLD-1 and LoVo cells arrested their growth, and this was accompanied by re-expression of PIK3CG. However, because we used a PI3K inhibitor and a 5-Aza demethylating agent, the data obtained in this study are indirect and

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* Grades of PIK3CG-positive cells were classified as high, low, and negative as described in the Text.

The correlation was analyzed by χ² test and Mann-Whitney U test. Ps less than 0.05 were regarded as statistically significant. N.S., not significant.

Histological typing, depth of invasion, and clinical stage were classified according to general rules for clinical and pathological studies on cancer of the colon, rectum, and anus (13). Well, well differentiated adenocarcinoma; Moderately, moderately differentiated adenocarcinoma; Poorly, poorly differentiated adenocarcinoma; m, mucosa; sm, submucosa; mp, muscularis propria; ss, subserosa; se, exposed subserosal invasion; a1 and a2, invasion to adventitia.

Ki-67 (MIB-1) labeling index (mean ± SD) was determined by counting the number of positive cells (%) in a total of 1000 or more tumor cells observed in 10 representative high-power fields (x400).

Grades of nuclear accumulation of β-catenin, overexpression of cyclin D1, and abnormal nuclear accumulation of p53 were classified as positive and negative as described in the Text.

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Fig. 5 Expression of PIK3CG in primary colon cancers. A, RT-PCR analysis of PIK3CG in human colon cancers. As a control, expression of HPRT is also shown. N, normal colon mucosa; T, tumor sample. B, the status of Cpg methylation of the promoter region of the PIK3CG gene in primary colon cancers and corresponding normal mucosa in case 2. When DNAs were digested with HpaII restriction enzyme, partial methylation of the promoter region of this gene was detected in the tumor (T) but not in the corresponding normal colonic mucosa (N).
phenomenological. Therefore, additional investigations, including a gene transfection assay, are needed to clarify the role(s) of PIK3CG in human colon cancers. Although blockade of the growth of human colon cancer cells by PIK3CG has been reported (11), Barbier et al. (24) showed that growth-factor-independent survival of HCT8/S11 cells was promoted by constitutively active, membrane-targeted PIK3CG, but not in its absence or after stable transfection with catalitically inactive PIK3CG. Further investigation to reveal the tumorigenic role of PIK3CG in human colon cancer cells is awaited.

In our present study, immunohistochemical examination revealed reduced PIK3CG expression in 85% of colorectal cancers, and this correlated with tumor invasiveness and metastasis. Some tumors exhibited markedly reduced PIK3CG levels at the front of their infiltrating regions (data not shown). Furthermore, particularly low levels of PIK3CG were frequently observed in poorly differentiated adenocarcinomas. Further investigation is needed to elucidate the correlation between reduction of the levels of this protein and poor differentiation of cancer cells. A recent study showed that the addition of the PI3K inhibitor LY294002 to the culture medium resulted in a rapid loss of MGP1 cell polarity, which suggested that the loss of PIK3CG may prevent gland formation and migration of colon cancer cells (25). It is interesting that the magnitude of the PIK3CG expression level reduction correlated significantly with the clinicopathological features, especially invasion depth and invasion of lymphatic vessels of the cancers. The Ras oncoprotein induces CD44 adhesion molecule cleavage through PI3K and the Rho family of small G proteins, which also suggests that PI3K participates in cell migration and invasion (26). Yeates et al. (27) reported that PIK3CG/PI3K activity may be the limiting factor in cells showing anchorage-independent growth, which could explain their increased sensitivity, compared with cells in monolayer culture, to inhibitors of PI3K signaling. These results support the hypothesis that a decrease in PIK3CG expression contributes to carcinoma invasiveness and/or metastasis rather than cell proliferation, especially in colorectal cancers, as indicated by our results.

Activation of Akt/PKB is initiated by phosphatidylinositol second messengers, and activated Akt/PKB subsequently mediates three distinct signals: cell survival signal, cell cycle entry, and cell differentiation and migration signals (1–4, 25–27). Activated Akt is likely to send survival signals by phosphorylating multiple targets, including BAD and caspase-9 (28, 29). Moreover, Akt catalyzes phosphorylation of GSK-3, resulting in β-catenin stabilization and translocation to the nucleus, which is followed by cyclin D1 overexpression in colon cancer cells (30, 31). Thus, PI3K may induce cell cycle entry in human mammary cells, including colon cancer cells. However, the data obtained in our present study did not indicate that the reduced expression of PIK3CG influences cell proliferation monitored by Ki-67 and p53.

The tumor suppressor PTEN inhibits Akt/PKB phosphorylation and blocks the PI3K-mediating pathway by converting the phosphatidylinositol second messengers (32, 33). Frequent somatic mutations of the PTEN gene, a negative regulator of the PI3K-Akt/PKB pathway, in sporadic endometrial carcinomas and brain tumors have been reported. These findings provide the evidence for the importance of the activation of the PI3K-Akt/PKB pathway in cellular transformation of these tumors. However, no mutation of the PTEN gene has been detected in human colorectal cancers (6), although we have reported the importance of the PI3K-Akt/PKB signaling pathway in colorectal cancers and have implied that PI3K inhibitors might lead to new therapeutic strategies (5, 14). We examined the effectiveness of the PI3K inhibitor LY294002 on the growth of colon carcinoma cells in vitro and in mouse xenograft models in vivo. Considering the fact that Akt/PKB is constitutively active in various human malignancies, we think that pharmacological agents such as LY294002 may prove to be of therapeutic benefit in the future.

REFERENCES
