Biological Function and Prognostic Significance of Peroxisome Proliferator-Activated Receptor delta in Rectal Cancer

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Relation of Peroxisome Proliferator-Activated Receptor δ Expression to Clinicopathologic and Prognosis of Rectal Cancer Patients

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Short title: PPAR δ role in rectal cancer

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This study is one part of a serial research on the role of PPAR δ in colorectal cancer.

Key words: peroxisome proliferator-activated receptor; rectal neoplasm; prognosis; pathogenesis; clinicopathological feature.

Translational Relevance

Peroxisome proliferator-activated receptor β/δ (PPAR β/δ) has been implicated in the pathogenesis of colorectal cancer, but its exact role remains controversial. This study demonstrates that PPAR δ is related to the early development of rectal cancer, and involved in the inhibition of the proliferation of colorectal cancer cells. These findings support the role of PPAR δ as a tumor suppressor and thus may be a good therapeutic target for colorectal cancer. In this study, we show that preoperative radiotherapy (RT) increases the PPAR δ expression in normal rectal mucosa while decreases it in primary rectal cancers and lymph node metastases. Therefore, application of a PPAR δ agonist or regulator together with preoperative RT in PPAR δ –low expression tumors may enhance the efficacy of RT. We show that radiotherapy decreases the PPAR δ expression in primary rectal cancers and lymph node metastases, and the increase of PPAR δ in primary rectal cancers is related to favorable survival of the patients, indicating that PPAR δ is a useful prognostic factor for rectal cancer patients.

Abstract

Purpose: To investigate the expression significance of peroxisome proliferator-activated receptor- β/δ (PPAR β/δ) in relation to radiotherapy, clinicopathological and prognostic variables of rectal cancer patients. Experimental **Design:** We included 141 primary rectal cancer patients who participated in a Swedish clinical trial of preoperative radiotherapy. Tissue microarray samples from the excised rectal cancers and the adjacent or distant normal mucosa and lymph node metastases were stained with PPAR δ antibody. Survival probability was computed by the Kaplan-Meier method and Cox regression model. The proliferation of colon cancer cell lines KM12C, KM12SM and KM12L4a was assayed after PPAR δ knockdown. **Results:** PPAR δ was increased from adjacent or distant normal mucosa to primary cancers while it decreased from primary cancers to lymph node metastases. After radiotherapy, PPAR δ was increased in normal mucosa while it decreased in primary cancers and lymph node metastases. In primary cancers, the high expression of PPAR δ was related to higher frequency of stage I cases, lower lymph node metastasis rate and low expression of Ki-67 in the cases without radiotherapy, and related to favorable survival in the cases either with or without radiotherapy. The proliferation of the KM12C, KM12SM or KM12L4a cells was significantly accelerated after PPAR δ knockdown. Conclusions: Radiotherapy decreases the PPAR δ expression in primary rectal cancers and lymph node metastases. PPAR δ is related to the early development of rectal cancer and inhibits the proliferation of colorectal cancer cells. Increase of PPAR δ predicts favorable survival in the rectal

cancer patients either with or without preoperative radiotherapy.

Introduction

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that are members of the nuclear hormone receptor superfamily, containing three isoforms, α , β/δ and γ (1). PPAR α and γ have been well-characterized as central regulators of lipid and glucose homoeostasis (2), but less is known about the biological roles of PPAR δ . Available studies indicate that, the major functions of PPAR δ are associated with its regulator roles in multiple biological processes, such as, lipid metabolism and energy homeostasis, embryo implantation, wound healing, inflammatory response, cell proliferation and carcinogenesis (3, 4). PPAR δ therefore represents a potential drug target for the treatment of some diseases such as obesity and metabolic syndrome, which has led to the development of several synthetic drug agonists (5).

Recent studies have implicated PPAR δ in the pathogenesis of colorectal cancer. Inactivation of adenomatous polyposis coli (APC) upregulates PPAR δ expression in colorectal cancer cells (6). PPAR δ is overexpressed in both human and rat colorectal cancers (7-9). Loss of PPAR δ expression in colon cancer cells results in decreased growth of xenografts (10). Ligand activation of PPAR δ potentiates colon tumorigenesis in mice (4, 11, 12). These studies support a promotive role of PPAR δ in colorectal carcinogenesis, but other studies conflict with these reports. Targeted deletion of APC alleles decreases PPAR δ expression in mouse intestine (13). PPAR δ expression is decreased in human colon cancers as compared to the matched normal

mucosa (14, 15). Ligand activation of PPAR δ attenuates colon tumorigenesis in mice (4, 16, 17). Experiments that examined polyp formation in PPAR δ -null APC min mice show either no effect (18), or paradoxically, an increase in polyp number and size compared with wild-type mice (13,16,17,19). Our recent study shows that PPAR δ may facilitate the differentiation of colon cancer cells (20). Collectively, the role of PPAR δ in colorectal carcinogenesis remains highly controversial.

Current studies on the role of PPAR δ in colorectal cancer are mostly based on cell lines or animal models, lacking clinical study with long-term observation data of patients. Little is known about the relationship of PPAR δ expression with radiotherapy (RT), clinicopathological and prognostic factors in colorectal cancer patients. In the present study, we examined the expression of PPAR δ in human rectal cancers and the matched adjacent or distant normal mucosa and lymph node metastases, with or without preoperative RT, by using immunohistochemistry (IHC). We analyzed the relationship between PPAR δ expression and preoperative RT, clinicopathological factors including survival of rectal cancer patients, and the proliferation of three colon cancer cell lines with different metastatic potentials. To our knowledge, this is the first study correlating PPAR δ expression with RT and prognosis in the rectal cancer patients who participated in a Swedish clinical trial of preoperative RT.

Materials and methods

Patients

This study included 141 primary rectal cancer patients from the Southeast Swedish Health Care region who participated in a randomized Swedish rectal cancer trial of preoperative RT between 1987 and 1990 (Swedish Rectal Cancer Trial, 1997) (21). The written informed consent was given by each participant. Seventy-seven patients received tumor resection alone, and 64 received preoperative RT followed by tumor resection. Locally curative resection was performed in all patients. Radiotherapy was given with 25 Gy in 5 fractions over a median of 6 days (range, 5–12 days), delivered with 6–10 MV photons. Surgery was then performed in a median of 3 days (range, 1–13 days) after RT. None of the patients received adjuvant chemotherapy. Table 1 presents the characteristics of the patients and tumors including gender, age, TNM stage (classified according to the Cancer Staging Manual of American Joint Committee on Cancer, 7th edition, 2010 [22]), grade of differentiation, number of other tumors, surgical type, resection margin, and mean distance to the anal verge. There was no statistical difference between the non-RT and RT groups regarding these characteristics (P > 0.05, Table 1).

Follow-up was performed by matching all patients against the Swedish Cancer Register and the Cause of Death Register until 2004. The median follow-up period was 84 months (range, 0–193 months). Information about local or distant recurrence, disease-free survival (DFS) and overall survival (OS) were obtained from patient medical records. We conducted our study after approval by the institutional review board of the Linköping University, Sweden.

Tissue collection and tissue microarray

Specimens were collected from primary rectal cancers (n = 141) and the matched adjacent normal mucosa (n = 81), distant normal mucosa (n = 115), and metastases in the regional lymph nodes (n = 36). Distant normal mucosa was taken from proximal or distal margin (4-35 cm from the primary tumor) of the resected rectum, and adjacent normal mucosa was from mucosa adjacent to the primary tumor, both were histologically free from pre-tumor and tumor. All the specimens including normal mucosa, primary cancers and lymph node metastases were paraffin-embedded for tissue microarray.

Representative paraffin-embedded tissue blocks were selected as donor blocks for the TMA. Three morphologically representative regions were chosen in each block and three cylindrical core tissue specimens (0.6 mm in diameter) were taken from these areas, inserted in a recipient paraffin block. Sections from this block were cut into 5µm chips using a microtome, mounted on microscope slides. The tissue microarrays were constructed using a manual arrayer (Beecher Inc., WI).

Immunohistochemical assay (IHC)

IHC staining for PPAR δ expression was done on 5-μm TMA sections from paraffin-embedded surgical specimens. The sections were deparaffinized in xylene, rehydrated in ethanol, and rinsed in distilled water. Masked epitope retrieval was done by boiling the sections in 1×DIVA buffer (Biocare Medical, CA) at 125°C for 30 seconds in a high-pressure cooker. Then, the sections were stored at room temperature for 20 min, followed by rinsing with PBS containing 0.5% bovine serum albumin.

The sections were incubated in 3% H₂O₂-methanol for 5 min to block the activity of endogenous peroxidase. After being washed in PBS, the sections were incubated with power block (Spring Bioscience, CA) for 10 min to reduce nonspecific background staining. The sections were incubated with the PPAR δ polyclonal rabbit anti-Human IgG (ARP37889, against N terminal, Aviva Systems Biology, CA) in a 1:400 dilution (2.5 µg/ml) with 5% skimmed milk PBS buffer over night. After being washed in PBS, the sections were incubated with a secondary antibody, Envision System Labelled Polymer-HRP Anti-Rabbit (Dakocytomation, CA) for 25 min. The sections were rinsed in PBS before reacting with 3, 3-diaminobenzidine tetrahydrochloride (ChemMate) for 8 min to produce coloration. Finally, the sections were counterstained with hematoxylin, followed by dehydration with ethanol. All steps were done at room temperature. Sections known to show positive staining for PPAR δ were included in each run, receiving either the primary antibody or PBS, as positive or negative controls. In all staining procedures, the positive controls showed clear staining, whereas there was no staining in the negative controls.

Measurements of PPAR δ expression by IHC

The IHC slides were examined independently in a blinded fashion by two investigators (L.Y. and H.Z.) without knowledge of clinicopathological or biological information. Each investigator estimated the proportion of cells stained and the intensity of staining in the whole section. The intensity in epithelial cells or tumor cells was scored as 0 (negative staining), 1 (weak staining exhibited as light yellow), 2 (moderate staining exhibited as yellow brown), and 3 (strong staining exhibited as

brown). The proportion of cells stained was accessed using a five scoring system: 0 (no positive cells), 1 (< 10 % positive cells), 2 (10 % - 40 % positive cells), 3 (40 % - 40 %70 % positive cells), and 4 (> 70 % positive cells). The percentage of cells at each intensity was multiplied by the corresponding intensity value to obtain an immunostaining score ranging from 0 to 12. The scores were combined to obtain an overall mean score. The cut-off value for expression levels from weak to strong was based on a measurement of heterogeneity using log-rank test statistical analysis with respect to overall survival. Using this assessment system, optimal cut-off values were identified by the mean score as follows: 0 (negative), 1~3 (weak), 4~9 (moderate), 9~12 (strong). If there was a discrepancy in individual scores, then both investigators re-evaluated the slides together to reach a consensus before combining the individual scores. To avoid an artificial effect, the cells on the margins of the sections and in areas with poor morphology were not counted. For statistical analysis, the negative or weakly stained cases were considered low expression and the moderate or strongly stained cases were considered high expression.

Evaluation of apoptosis, proliferation and phosphatase of regenerating liver-3 (PRL-3), cyclooxygenase-2 (COX-2) and survivin

The data for apoptosis, detected by terminal deoxynucleotide transferase-mediated deoxyuridine triphosphate—biotin nick end labeling (TUNEL) assay (n=136), proliferation (n=126), PRL-3 (n=125), COX-2 (n=138) and survivin (n=98) of primary rectal cancers determined by IHC, were taken from previous studies

performed with the same cases used in the present study at our laboratory (23-27). The percentage of apoptotic cancer cells was determined by counting approximately 1000 tumor cells. Cases were considered as negative if apoptotic cells constitute <5% of tumor cells. Proliferation in the cancer cells was measured using IHC for Ki-67 as an indicator. Low proliferation was defined in sections where <30% of cancer cells expressed Ki-67 and high proliferation in section where Ki-67 was expressed in ≥30% of cancer cells.

Western blot assay

Total proteins from the normal rectal mucosa samples, primary rectal cancers or lymph node metastases were extracted using $1\times$ RIPA lysis buffer (Santa Cruz, CA) and the concentrations were assayed by the Bicinchoninic acid Protein Assay (Pierce, IL). Each 20 µg aliquot of total proteins was loaded in duplicate in 10% SDS-PAGE gel, and then transferred onto a 0.2 µm polyvinylidene diffuoride membrane (Bio-Rad, CA). After completing protein transfer, the membrane was blocked in 5% (w/v) skimmed milk in PBS and incubated overnight with the rabbit polyclonal antibody (IgG) against PPAR δ (0.3µg /ml, Aviva Systems Biology, CA), COX-2 (0.5 µg /ml; Catalogue No. ab52237, Abcam), or survivin (1.0 µg/ml; Catalogue No. ab8228, Abcam), respectively. The blots were detected by the secondary antibody, horseradish peroxidase (HRP)-linked polyclonal goat anti-rabbit IgG (0.1 µg/ml, DakoCytomation, Denmark) and visualized with Amersham ECL plus Western blot detection system (Amersham Biosciences/GE Healthcare, UK). Protein expression was normalized to the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) detected by the

rabbit monoclonal antibody (HRP conjugated, 0.04 µg/ml; Cell Signaling, MA).

Cell culture

Three human colon cancer cell lines, KM12C, KM12SM and KM12L4a (from Professor IJ Fidler, Anderson Cancer Center, TX), untreated or treated by lentivirus targeting PPAR δ gene (Lenti-PPAR δ) or by lentivirus without RNA interference effect (Lenti-control) from our previous study (20), were used. All the cell lines were maintained in Eagle's minimal essential medium (MEM) with Earle's salts, l-glutamine and nonessential amino acids (Sigma-Aldrich), supplemented with 1.5% NaHCO3, 1 mm Na-Pyruvate (Invitrogen, Carlsbad, CA, USA), 1 × MEM Vitamin Solution (Invitrogen), 1 % Penicillin-Streptomycin (Invitrogen), 1 μ g/ml Puromycin (only for the treated cells to maintain the purity) and 10 % fetal bovine serum (Invitrogen). The expression of PPAR δ had been stably silenced in the cells treated by Lenti-PPAR δ while remaining unaffected in the Lenti-control treated cells, as confirmed in our previous study (20) (see Supplemental Data). Of the three cell lines, KM12C has poor metastatic potential while KM12SM and KM12L4a have high metastatic potentials (28).

Cell proliferation assay

The effect of PPAR δ knockdown on the proliferation of KM12C, KM12SM and KM12L4a cells was assayed with MTT (methyl thiazolyl tetrazolium). The log-phase cells, untreated or treated with Lenti-PPAR δ or Lenti-control, were seeded in 96-well plates (4×10³/well) for 24-96 h. Each group was analyzed every 24 h in triplicate by the following method: each well had 20 μ l MTT (5 mg/ml, Sigma-Aldrich, Sweden)

added and were incubated for a further 4 h at 37 °C; subsequently the formazan crystals were solubilized with 150 µl dimethyl sulphoxide (DMSO, Sigma-Aldrich). The absorbance (A) value was measured at 570 nm wavelength on an automatic Microplate Reader (model 550, Bio-Rad) with DMSO as the blank. The growth curve of cells was drawn using growth date as X-axis and A value as Y-axis.

Statistical analysis

All statistical analyses were performed using STATISTICA software package (version 7.0; STATSOFT Inc, Tulsa, OK). McNemar's or Pearson Chi-square methods were used to test the significance of the differences in PPAR δ expression between the adjacent or distant normal mucosa, primary cancers and lymph node metastases, and the association of PPAR δ expression with clinicopathologic variables. The relationship between PPAR δ expression and survival, local recurrence or distant recurrence was tested using Kaplan-Meier analysis (Log rank test) and Cox proportional hazards regression analysis (likelihood ratio test). Analysis of variance (ANOVA) and post hoc multiple comparison (LSD, two-tailed) was applied to test the quantitative analysis in Western blot assay and the difference of absorbance values in MTT assay. The test was two-sided and a P value of less than 0.05 was considered statistically significant.

Results

PPAR δ was increased from normal mucosa to primary cancers, while it decreased from primary cancers to lymph node metastases

By IHC, PPAR δ was detected predominantly in the cytoplasm of epithelial cells in normal mucosa, and tumor cells of primary cancers and lymph node metastases, with a little staining in the nuclei (Fig. 1A). For the further analyses of this study, only the staining of cytoplasmic PPAR δ was measured and presented. In the cases without RT, the frequency of high expression (moderate or strong staining) of PPAR δ was significantly increased from adjacent normal mucosa (2%, 1 of 44) or distant normal mucosa (10%, 6 of 61) to primary cancers (61%, 47 of 77), while expression decreased from primary cancers to lymph node metastases (32%, 9 of 28; P = 0.008; Fig. 1B). There wasn't a significant difference between adjacent and distant normal mucosa (P = 0.23).

The specifity of the PPAR δ antibody used in IHC was examined by Western blot. As shown in Fig.1C, each of the electrophoretic lanes had a clear band at the expected position for PPAR δ (48 kDa) as well as the band for GAPDH (37 kDa). Quantitative analysis showed that, the expression of PPAR δ protein normalized by GAPDH was significantly increased by 64.0 ± 2.6 % from normal mucosa to primary cancers, and decreased by 10.8 ± 1.3 % from primary cancers to lymph node metastases (P = 0.036; Fig. 1C), which was identical to the results of IHC.

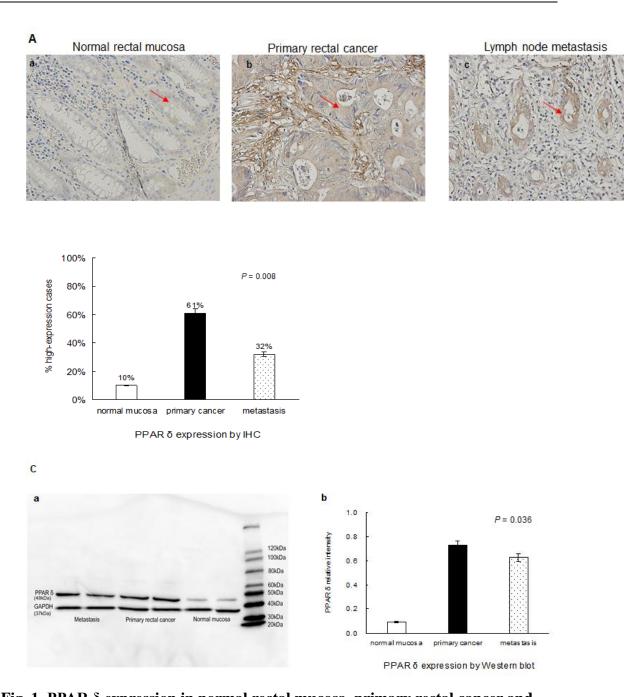


Fig. 1. PPAR δ expression in normal rectal mucosa, primary rectal cancer and lymph node metastasis. A. By immunohistochemical assay (IHC), PPAR δ was predominantly detected in the cytoplasm (red arrow) of epithelial cells in normal mucosa (a), and tumor cells of primary cancer (b) and lymph node metastasis (c), with a little staining in the nuclei. All images were taken at a magnification of \times 400. B. The frequency of the cases with high expression (moderate or strong staining) of PPAR δ increased from normal mucosa samples to primary cancers, and decreased from primary cancers to lymph node metastases (P = 0.008). C. Western blot analysis showed that, (a) the expression of PPAR δ was obviously higher in primary cancers or lymph node metastases than in normal mucosa samples; (b) Quantitative analysis showed that the expression alteration of PPAR δ protein was identical with that of the IHC staining.

PPAR δ was increased in normal mucosa while it decreased in primary cancers and lymph node metastases after RT

Compared with the unirradiated cases, the frequency of highly-expressed PPAR δ in irradiated cases was significantly increased in both adjacent normal mucosa (2% vs. 16%; P < 0.001) and distant normal mucosa (10% vs. 33%; P = 0.014), while it decreased in primary cancers (61% vs.41 %; P = 0.031) and in lymph node metastases (32% vs.22%; P = 0.043; Fig. 2).

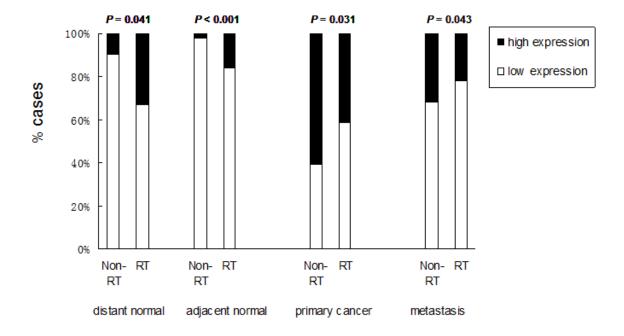


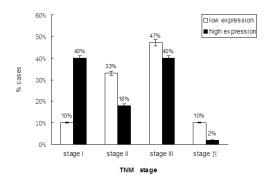
Fig. 2

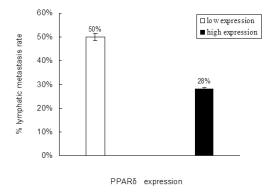
Fig. 2. The influence of radiotherapy (RT) on PPAR δ expression. After RT, the frequency of highly-expressed PPAR δ was significantly increased from 2 % to 16 % in adjacent normal mucosa (P < 0.001, 95% CI: 0.14~0.26) and from 10 % to 33 % in distant normal mucosa (P = 0.014, 95% CI: 0.30~0.47), while it decreased in primary cancers from 61 % to 41 % (P = 0.031, 95% CI: 0.35~0.50) and from 32 % to 22% in lymph node metastases (P = 0.043, 95% CI: 0.19~0.30). CI: confidence interval.

PPAR δ expression in primary cancers was related to TNM stage, lymph node metastasis, Ki-67 expression and differentiation

In the primary cancers without RT, the high expression of PPAR δ was associated with higher frequency of stage I cases and lower frequency of stage II, III and IV cases (P = 0.015; Fig. 3A), and related to lower rate of lymph node metastasis (P = 0.046; Fig. 3B), compared with the cases with low expression (negative or weak staining) of PPAR δ . After RT, the relationship between PPAR δ expression and TNM stage (P = 0.43) or lymph node metastasis disappeared (P = 0.27; data not shown).

In the cases either with or without RT, the high expression of PPAR δ in primary cancers was related to low expression (<30% of cancer cells) of Ki-67 (P = 0.016, 0.025; Fig. 3C). The non-RT cancers with better differentiation showed a significantly higher frequency of highly-expressed PPAR δ than did those with poor differentiation (P = 0.004; data shown in our previous study [20]). There was no significant relationship between PPAR δ expression and age, gender, growth pattern, apoptosis, PRL-3, COX-2 or survivin (P > 0.05; data not shown). The Western blot assay confirmed that the expression of PPAR δ wasn't significantly different from the expression of COX-2 or survivin in primary rectal cancers (P = 0.43; see Supplemental Data).





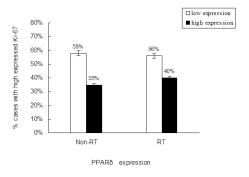


Fig. 3. The relationship between PPAR δ expression in primary rectal cancers and TNM stage, lymph node metastasis and Ki-67 expression. (A~B) the cases were unirradiated. The cases with high expression (moderate or strong staining) of PPAR δ exhibited (A) higher frequency of stage I cases and lower frequency of stage II, III and IV cases (P = 0.015) and (B) lower rate of lymph node metastasis (P = 0.046), compared with those with low PPAR δ expression (negative or weak staining). (C) Both the non-RT and RT cases demonstrated a negative correlation of PPAR δ expression with the expression of Ki-67 (P = 0.016, 0.025).

High expression of PPAR δ in primary cancers was related to favorable survival, without relation to local/distant recurrence

Kaplan-Meier analysis demonstrated that the high expression of PPAR δ in the unirradiated primary cancers was related to better DFS (P=0.003; Fig. 4A) but not OS (P=0.13, data not shown). In the irradiated cases, the high expression of PPAR δ was related to better OS (P=0.032; Fig. 4B) but not DFS (P=0.10, data not shown). In multivariate analysis, the above significances still remained, independent of age, gender, tumor differentiation, growth pattern and TNM stage (Table 2). As shown in Table 2, the unirradiated patients with low expression of PPAR δ were 3.6 times more likely to die of rectal cancer than those with high expression of PPAR δ (hazard ratio [HR] 3.6, P=0.042; 95% confidence interval [CI], 1.1~14.7). The irradiated patients with low expression of PPAR δ were 4.9 times more likely to die than those with high expression of PPAR δ (HR 4.9, P=0.029; 95% CI, 1.8~12.1).

In the cases either with or without RT, the expression of PPAR δ in primary cancers wasn't significantly related to local recurrence or distant recurrence, although the cases with high expression of PPAR δ tended to have delayed time to local or distant recurrence (P > 0.05; see Supplemental Data).

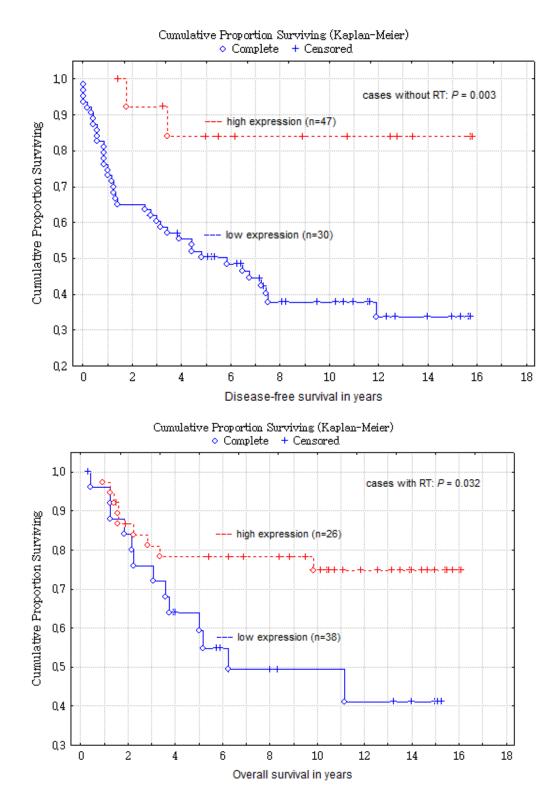
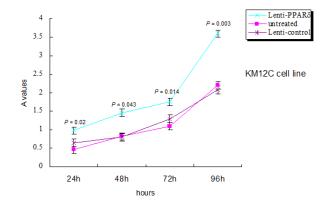
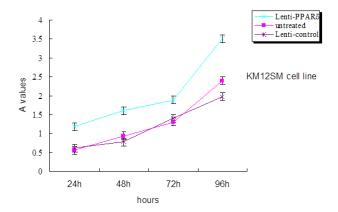


Fig. 4. The relationship between PPAR δ expression in primary rectal cancers and survival and local/distant recurrence. (A) Unirradiated patients with high expression of PPAR δ in primary rectal cancers exhibited better disease-free survival, compared with low expression cases (P = 0.003). (B) Irradiated patients with high expression of PPAR δ in primary rectal cancers had better overall survival, compared with low expression cases (P = 0.032).

Knockdown of PPAR δ promoted the proliferation of KM12C, KM12SM and KM12L4a cells

By MTT assay, the colon cancer cells, KM12C, KM12SM and KM12L4a with silenced PPAR δ , exhibited a significant increase in proliferation (A value) at each time point, compared with untreated cells and the control cells treated with the Lenti-control (P < 0.05). There was no significant difference between untreated cells and control cells (P > 0.05; Fig. 5). The comparison of the A values at each time point among the three cell lines didn't show significant difference (P > 0.05; data not shown).





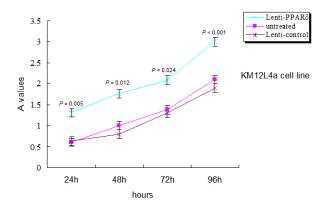


Fig. 5. Cell proliferation curve of the KM12C, KM12SM and KM12L4a cells after knockdown of PPAR δ. The proliferation of KM12C, KM12SM and KM12L4a cells, treated by lentivirus against PPAR δ (Lenti-PPAR δ) or by lentivirus without RNAi effect (Lenti-control) and untreated ones (untreated), were assayed by MTT. The group with silenced PPAR δ showed a significantly increased A value over 96 h incubation compared to untreated or Lenti-control treated group at each time point, in all the three cell lines (A) KM12C, (B) KM12SM and (C) KM12L4a (P < 0.05).

Discussion

In the present study, we found that PPAR δ was predominantly located in the cytoplasm of the epithelial cells in normal mucosa, and tumor cells of primary cancers and lymph node metastases, with little expression in the nuclei. This finding is consistent with the studies by Takayama et al. (8) and Yoshinaga et al. (29). The specificity of the PPAR δ antibody in the present study was verified by Western blot, which showed a clear band at the expected position of PPAR δ protein as shown in Figure 1C. The cytoplasmic accumulation of PPAR δ in human rectal tissue may be necessary for the proteins to be available for their nuclear role whenever required. Known as a nuclear receptor, PPAR δ was found to be located mainly in the nuclei of colorectal cancer cells (7, 30). However, these studies couldn't be reproduced by Western blot as reported by Foreman et al. (31). Future studies need to confirm the location of PPAR δ protein in colorectal cancer cells, by Western blot comparing nuclear extracts with cytoplasmic fractions.

The expression patterns of PPAR δ in colorectal cancers have been reported, but the majority of available data aren't supported by Western blot. For example, He et al (6) and Gupta et al (32) observed the overexpression of PPAR δ mRNA in human colorectal cancer tissues by Northern blots. IHC analysis showed the increase of PPAR δ expression in human colon cancers but no quantified analysis of Western blot was provided (8). Another report suggested that expression of PPAR δ was higher in flat dysplastic adenomas from Apc min heterozygous mice while Western blot analysis showed no change in the expression of PPAR δ in adenomas as compared to normal

mucosa (30). To date, few studies have reported the differential expression of PPAR δ between primary cancers and lymph node metastases. In the present study, we used both IHC and Western blot to quantitate the expression of PPAR δ in normal rectal mucosa, primary cancers and lymph node metastases. Both IHC and Western blot showed that PPAR δ was increased in primary cancers compared to adjacent or distant normal mucosa, and then deceased from primary cancers to lymph node metastases. Our finding confirms that PPAR δ may be involved in the pathogenesis of rectal cancer, and indicates that PPAR δ is related to the lymph node metastasis.

We analyzed the influence of RT on the PPAR δ expression and found that, PPAR δ was increased in the adjacent or distant normal mucosa samples, and decreased in primary cancers and lymph node metastases after RT. Increase of PPAR δ has recently been reported to protect cells from stress-induced injury, oxidation and DNA damage (33, 34). In this regard, the increase of PPAR δ in the normal mucosa samples may be a protective response to RT, and its decrease in primary cancers or lymph node metastases seems harmful to tumor cells. However, given the suppressor role of PPAR δ in rectal cancer as discussed later, its decrease in rectal cancers may compromise the therapeutical efficacy of RT.

Takayama et al. (8) analyzed the relationship between PPAR δ expression and clinicopathological factors of colorectal cancer by IHC, but didn't find any difference. As mentioned in their report, the small number of specimens (32 cases) may have given a low statistical power. In the present study, we found that the high expression of PPAR δ in non-RT cases was related to higher frequency of stage I cases and lower

frequency of lymph node metastasis. These findings indicate that PPAR δ may be an early event in the development of rectal cancer and may be involved in the lymph node metastasis. Therefore, the examination of PPAR δ in rectal cancer may be valuable for the preoperative evaluation, which may help in deciding treatment protocol for patients.

In the present study, we observed a significant association between increased PPAR δ and favorable patient survival in primary rectal cancers. The increase of PPAR δ in primary cancers was significantly related to better DFS in the cases without RT and better OS in those with RT. Ishizuka et al reported that increased PPAR δ was associated with favorable postoperative OS in the patients with colorectal cancers (35). However, that study used small samples (26 cases) in a short observation period (mean 32.3 months) and had no the information about RT. The present finding suggests that, high expression of PPAR δ is an independent indicator of a good prognosis for the rectal cancer patients undergoing surgery alone or surgery plus preoperative RT. We observed that the cases with highly-expressed PPAR δ tended to have delayed time to local/distant recurrence though the differences did not reach statistical significance. This result seems inconsistent with the relationship between increased PPAR δ and favorable patient survival observed in this study. The non-significance between the PPAR δ expression and local/distant recurrence may be explained by the relatively small sample size in this study, which has confined the statistical significance. In addition, the expression of PPAR δ may be associated with some unknown factors, which affected the survival of the patients more strongly than

local/distant recurrence.

To explore the mechanisms underlying our findings, we further analyzed the relationships of PPAR δ with apoptosis, Ki-67, PRL-3, COX-2 and survivin in the primary cancers. Ki-67 is a proven indicator of cell proliferation (36). COX-2 has been shown to participate in the apoptosis inhibition, angiogenesis, cell invasion and metastasis of colorectal cancer (37). Survivin is a member of the apoptosis inhibitors (38). PRL-3 has been identified as an important protein in the metastatic process of colorectal cancer (26). We found that increased PPAR δ was significantly related to the decreased Ki-67 in either non-RT or RT cases, but not to apoptosis, PRL-3, COX-2 or survivin. This finding indicates that PPAR δ may be involved in the cell proliferation of rectal cancer, without participation in the physiological process of apoptosis, PRL-3, COX-2 and survivin.

Further assays showed that all the three cell lines, KM12C, KM12SM and KM12L4a, exhibited increased proliferation after PPAR δ knockdown, indicating an inhibiting role of PPAR δ on the proliferation of colon cancer cells. This finding confirms our previous observation that the silencing of PPAR δ significantly promoted the proliferation of HCT-116 cells (39). We have recently shown the promotional role of PPAR δ in the differentiation of both colon cancer cell lines and tissue (20). Taken together, the present findings demonstrate that PPAR δ plays an inhibiting role in the progression of colorectal cancer, the mechanism underlying which is associated with its functions of inhibiting the proliferation and promoting the differentiation of cancer cells. Consistent with our findings, recent studies have provided evidence to support

the inhibiting role of PPAR δ in colorectal carcinogenesis (13,16, 17, 19). The inhibiting role of PPAR δ in colorectal cancer is consistent with the other findings in the present study, where increased PPAR δ is related to a higher frequency of early stage tumors, a lower rate of lymph node metastasis and favorable survival of rectal cancer patients. We didn't find a significant difference in cellular proliferation among the KM12C, KM12SM and KM12L4a cells after PPAR δ knockdown. This indicates that the effect of PPAR δ on the proliferation of colon cancer cells was not associated with the cell metastatic potentials.

Compared with ordinary IHC sections, TMA technology greatly improves internal experimental control as it allows simultaneous staining of massive sections. The present study benefits from the advantages of TMA, however the use of only three core tissue specimens per block when preparing TMA might lead to a limitation of reprehensive samples due to tumor heterogeneity.

In conclusion, increased PPAR δ in primary rectal cancers is related to higher frequency of early stage tumors and lower rate of lymph node metastasis. PPAR δ plays a role in inhibiting the proliferation of colorectal cancer cells. These findings demonstrate that PPAR δ is related to the early development of rectal cancer and support the role of PPAR δ as a tumor suppressor in colorectal carcinogenesis. We show that preoperative RT increases the PPAR δ expression in normal mucosa while decreasing it in primary rectal cancers and lymph node metastases. Therefore, the application of a PPAR δ agonist or regulator together with preoperative RT in PPAR δ -low expression tumors may enhance the effect of RT. We show that increased PPAR

 δ in primary rectal cancers is independently related to a favorable survival of patients, demonstrating that PPAR δ is a useful prognostic factor for rectal cancer patients.

Disclosure of Potential Conflict of Interest

No potential conflicts of interest were disclosed.

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Table 1. Characteristics of patients and tumors

Characteristics	Radiotherapy	Non-radiotherapy	P^{\dagger} value	
Characteristics	% (n)	% (n)	P' value	
Gender			0.58	
Male	42 (66)	45 (58)		
Female	22 (34)	32 (42)		
Age (years)			0.89	
< 65	24 (38)	28 (36)		
≥ 65	40 (62)	49 (64)		
TNM stages			0.86	
I + II	40 (63)	40 (52)		
III + IV	24 (37)	37 (48)		
Tumor differentiation			0.14	
Good	7 (11)	5 (6)		
Moderate	39 (61)	54 (70)		
Poor	18 (28)	18 (24)		
Number of tumors			0.64	
Single	59 (92)	70 (91)		
Multiple *	4 (6)	5 (6)		
Unknown	1 (2)	2 (3)		
Surgical type			0.76	
Anterior resection	31 (48)	40 (52)		

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Abdominoperineal	33 (52)	37 (48)	
To anal verge (cm)			0.94
Mean	8.5	7.6	

^{*} There had been previous colorectal cancer and/or other types of tumors before the present rectal cancer. † Pearson Chi-square test.

Table 2. Cox multivariate analysis assessing the prognostic significance of cytoplasmic PPAR δ expression in primary tumor

Variables			Indicator of poor survival*	HR [†] (95% CI [‡])	P^{\S}
PPAR δ	No-RT	high vs. low	low	3.6 (1.1~14.7)	0.042
expression	RT	high vs. low	low	4.9 (1.8~12.1)	0.029
Age	No-RT	< 65 vs. ≥ 65	> 65	1.9 (1.1 ~3.6)	0.041
	RT		≥ 65	1.2 (0.9 ~5.2)	0.063
Gender	No-RT	female vs. male	vs. male male	0.2 (0.4 ~3.2)	0.39
	RT			0.3 (1.0 ~6.1)	0.73
Tumor	No-RT	(good + moderate)	poor	2.1 (1.7~13.5)	0.02
grade	RT	vs. poor		1.1 (0.6~11.9)	0.19
Growth	No-RT	Infiltrative vs.		1.2 (1.8~2.4)	0.36
pattern	RT	expansive	infiltrative	0.5 (0.2~1.7)	0.45
TNM stage	No-RT	(I · II) (III · III)	III+IV	14.3 (3.5 ~ 34.6)	< 0.001
	RT	(I + II) vs. (III+IV)		24.2 (4.6 ~ 23.7)	< 0.001

^{*}The analysis of disease-free survival is presented for the cases without RT, while overall survival is for the cases with RT; †hazard ratio; ‡confidence interval; \$significance was analyzed by Cox regression model.