Clinical implication of expression of cyclooxygenase-2 and peroxisome proliferator activated-receptor γ in epithelial ovarian tumours

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Expression of cyclooxygenase (COX)-2 plays a key role in tumorigenesis and development and peroxisome proliferator-activated receptor γ (PPARγ) has been implicated in the control of COX-2 expression in some tissues. The aim of this study is to investigate (1) whether expression of COX-2 and PPARγ is associated with ovarian carcinogenesis and progression of ovarian tumours and (2) whether COX-2 expression is controlled through ligand-mediated activation of PPARγ in ovarian carcinoma cells. For this purpose, the presence of COX-2 and PPARγ was immunohistochemically examined in 71 epithelial ovarian carcinomas, 18 borderline tumours and 23 benign tumours and the levels of COX-2 and PPARγ proteins were determined by enzyme immunoassay in four benign tumours, three borderline tumours and 12 carcinomas. The frequency of COX-2 and PPARγ detection was significantly increased and decreased as lesions progressed to carcinoma, respectively. The COX-2 protein was not detected in the three borderline tumours, whereas PPARγ protein was detected in all of them. COX-2 protein was detected in eight of the 12 carcinomas, whereas PPARγ protein was detected in only two cases. In addition, PPARγ protein was not detected in all of the eight carcinomas in which COX-2 protein was detected, suggesting that expression of PPARγ and COX-2 was in a reciprocal relationship. Furthermore, in cultured ovarian carcinoma cells, Western blot revealed that PPARγ and COX-2 expression was regulated conversely as a result of stimulation by 15-deoxy-Δ12, 14 PGJ2 (15-PGJ2), a PPARγ activator. In addition, 15d-PGJ2 suppressed tumour necrosis factor-α-induced-COX-2 expression, confirming the reciprocal correlation between COX-2 and PPARγ. From these results, it was suggested that PPARγ activation might suppress COX-2 expression via the nuclear factor-κB pathway in the ovarian carcinoma cells and that low expression of PPARγ and high expression of COX-2 might be involved in carcinogenesis and progression of ovarian tumours.

Keywords: COX-2; PPARγ; ovarian tumour; carcinogenesis; 15d-PGJ2

Cyclooxygenase (COX) is a rate-limiting enzyme in prostaglandin (PG) synthesis because of its rapid autoinactivation (Smith and Marnett, 1991). COX has two isoforms, the constitutive COX-1 and the inducible COX-2 (O’Banion et al, 1992). COX-1 is expressed in most tissues, whereas COX-2 is largely absent but is responsible primarily for PGs produced in inflammatory sites, suggesting that COX-2 plays a critical role in inflammation (Smith et al, 1996). Epidemiological studies have shown 40–50% reduction in mortality from colorectal carcinoma in continuous users of nonsteroid anti-inflammatory drugs (NSAIDs) compared with that of noncontinuous users (Thun et al, 1991; Giovannucci et al, 1995). Antitumour effect of NSAIDs was caused by inhibition of COX-2 (Taketo, 1998). COX-2 is known to be a promoter of gastrointestinal carcinoma. Many studies suggest that overexpression of COX-2 might be involved in multistep carcinogenesis and tumour progression especially in gastric and colorectal carcinoma (Prescott and White, 1996; van Rees et al, 2002). Recently, a progressive development from ovarian serous tumours of low potential malignancy to invasive serous carcinoma has been suggested in parallel to the concept of adenoma–carcinoma sequence in colorectal carcinomas (Hauptmann and Dietel, 2001), although the concept that ovarian carcinogenesis is a linear pathway as for colorectal carcinoma is still contentious. Shigemasa et al (2003) reported that COX-2 expression might play an important role in ovarian carcinoma development. Ferrandina et al (2002) described that increased COX-2 expression was associated with chemotherapy resistance and outcome in ovarian carcinoma patients and then Denkert et al (2002) reported that COX-2 expression was an independent prognostic factor in ovarian carcinoma. More recently, COX-1 has been reported to contribute to carcinoma development in the ovary through stimulation of neovascularisation (Gupta et al, 2003).

Peroxisome proliferator-activated receptor γ (PPARγ) is a member of a nuclear hormone receptor superfamily that can medulate gene expression upon ligand binding (Subbaramaiah et al, 2001). Ligand-mediated activation of PPARγ has been linked to cellular differentiation, apoptosis and anti-inflammatory responses. In colon carcinoma cells, ligand activation of the receptor inhibits cell growth, induces a differentiation response and reverses the malignant phenotype (Sarraf et al, 1998). PPARγ agonists and PPARγ overexpression led to a drastic reduction of the cell growth rate in PPARγ-expressing thyroid carcinoma cells (Martelli et al, 2002).

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(15d-PGJ2) was identified as a potent natural ligand for the PPARγ (Forman et al, 1995; Kliewer et al, 1995). 15d-PGJ2 was found to induce apoptosis, inhibit proliferation and prevent the growth of human breast carcinoma cells in the nude mouse model (Clay et al, 1999). Inhibition of COX-2 and activation of PPARγ inhibited the development of rat mammary gland carcinogenesis (Suh et al, 1999; Harris et al, 2000). Additionally, independent studies showed that COX-2 and PPARγ are induced and inactivated, respectively, in human breast carcinoma (Jiang et al, 2000; Ristimaki et al, 2002). Inoue et al (2000) proposed that expression of COX-2 was regulated by a negative feedback loop mediated through PPARγ in macrophages. Badawi and Badr (2003) described that the altered expression of COX-2 and PPARγ might influence the development of human breast carcinoma and its progression to metastasis. Recently, PPARγ has been reported to be localised primarily to granulosa cells in ovarian tissue and to be involved in follicular development (Komar et al, 2001).

The present study was designed to investigate whether expression of COX-2 and PPARγ is associated with ovarian carcinogenesis and progression of ovarian tumours. We also examined whether COX-2 expression was controlled through ligand-mediated activation of PPARγ in ovarian carcinoma cells. This is the first report describing the relationship between expression of COX-2 and PPARγ in ovarian malignancies.

MATERIALS AND METHODS

Study population and tissues

Immunohistochemical examination was performed retrospectively on 112 epithelial ovarian tumours obtained from women who were surgically treated at the Hirosaki University Hospital between 1989 and 2003 after informed consent had been obtained. The tissue specimens included 71 carcinomas, 18 borderline tumours and 23 benign cystadenomas. All patients with epithelial ovarian carcinoma were surgically staged in accordance with the 1988 International Federation of Gynecology and Obstetrics (FIGO) criteria. Patients included in this study had not received any preoperative chemotherapy. The breakdown for stages of ovarian carcinomas consisted of 40 patients with stage I, seven with stage II, 18 with stage III, six with stage IV. Histological types were classified into 30 cases with serous cystadenocarcinoma, 11 with mucinous cystadenocarcinoma, 17 with endometrioid adenocarcinoma, 12 with clear cell adenocarcinoma and one with undifferentiated adenocarcinoma. All patients with ovarian carcinoma received postoperative chemotherapy combining cisplatin (60 mg m⁻²), epirubicin (40 mg m⁻²) and cyclophosphamide (300 mg m⁻²). The duration of follow-up ranged from 8 to 156 months (median, 54 months). The mean age of patients with ovarian carcinoma at surgery was 54.1 years (range, 28 – 78 years). Of the 18 borderline tumours, four were serous and 14 mucinous. Of the 23 benign cystadenomas, nine were serous and 14 mucinous.

Immunohistochemical staining of COX-2 and PPARγ

Anti-COX-2 (Immuno-Biological Laboratories, Gunma, Japan) and anti-PPARγ (Cayman Chemical, Ann Arbor, MI, USA) antibodies were used at a concentration of 5 μg ml⁻¹, respectively. All samples surgically obtained for immunohistochemistry were immediately fixed in formaldehyde and embedded in paraffin. Sections 6 μm thick were routinely processed through xylene and a graded alcohol series and placed in 0.01 M citrate buffer, pH 6.0, and heated at 500 W in a microwave oven for 5 min to retrieve tissue antigen. The sections were treated with 0.3% hydrogen peroxide (H₂O₂) in methanol for 10 min to quench the endogenous peroxidase activity within the tissue. Nonspecific binding sites were blocked with 1% bovine serum albumin (BSA) and 20% heat-inactivated goat serum in phosphate-buffered saline (PBS) for 30 min at room temperature (RT). The sections were then stained for COX-2 or PPARγ by the avidin–biotin–peroxidase complex method using the appropriate antibodies as reported previously (Yokoyama et al, 2003). Anti-COX-2 antibody was applied for 12 h at 4 °C in a moist chamber. Anti-PPARγ antibody was applied for 1 h at 37 °C. The binding sites of peroxidase were visualized with 0.02% diaminobenzidine (DAB) (Sigma-Aldrich, St Louis, MO, USA) as a chromogen in Tris-HCl buffer, pH 7.6 containing 0.03% H₂O₂. The sections were then counterstained with haematoxylin for microscopic examination. As negative control, preimmune rabbit serum was used instead of the antibody. As positive control for COX-2 and PPARγ staining, formalin-fixed paraffin-embedded sections of colon carcinoma and urinary bladder carcinoma were stained by the same procedure, respectively. Two observers (AS and YY) independently evaluated and interpreted the results of immunohistochemical staining, without the knowledge of the clinical data of each patient. Cases in which more than 10% of tumour cells were as strongly immunoreactive as positive control cells were considered positive.

Measurement of levels of COX-2 and PPARγ

Analysis of levels of COX-2 and PPARγ in the ovarian tumours was carried out by enzyme immunoassay (EIA). Four benign tumours, three borderline tumours and 12 carcinomas kept at −80 °C were used for this experiment. For measurement of COX-2 and PPARγ levels, the tissue specimens (100 mg) were minced, sonicated in 10 ml Tris-HCl, pH 7.4, 0.5% NaCl and centrifuged at 10000 g for 15 min at 4 °C to separate cell debris and the fat layer. The resulting supernatants were used for analysis. EIA for COX-2 and PPARγ was carried out using a human COX-2 assay kit (Immuno-Biological Laboratories, Gunma, Japan) and a TransAM™ PPARγ Transcription Factor Assay kit (Active Motif, Carlsbad, NM, USA), respectively, according to each manufacturer’s instruction. Colour intensity was measured at 450 nm using BIORAD Model 550 microplate reader (Bio-Rad Laboratories, Tokyo, Japan) and the levels of COX-2 and PPARγ were expressed as ng per mg protein and μg per cell extract per well, respectively. The range of measurement sensitivity in COX-2 and PPARγ levels is 2.15 to 275 ng ml⁻¹ and 0.75 to 7.5 μg (cell extract)⁻¹ (well⁻¹), respectively. Standard curves and positive and negative controls were generated for COX-2 and PPARγ and assayed simultaneously with the samples.

Cell culture

OVCAR-3 cells were obtained from the American Type Culture Collection and the ovarian carcinoma cell line, which was derived from serous adenocarcinoma and was established at the Hirosaki University Hospital, was used. Cells were seeded at 1.2 × 10⁴ cells cm⁻² and grown in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U ml⁻¹ penicillin and 100 mg ml⁻¹ streptomycin, at 37 °C in a water-saturated atmosphere with 5% CO₂/95% air.

15d-PGJ2 treatment

After 24 h, the medium was replaced by the fresh medium (10% FBS) containing 15d-PGJ2 (Alexis Biochemicals, San Diego, CA, USA) at a final concentration of 0.1, 1, 10 or 20 μM. Cells were exposed to 15d-PGJ2 at the indicated concentrations for 72 h of treatment. As a negative control, cells were cultured in a medium without 15d-PGJ2. For each cell culture set, expression of COX-2 and PPARγ in 15d-PGJ2-treated and untreated cells was examined using Western blot analysis.

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Tumour necrosis factor-α and 15d-PGJ2 treatment

The medium was replaced with serum-free medium 12 h before stimulation. OVCAR-3 cells were then treated with 20 ng ml⁻¹ tumour necrosis factor (TNF)-α (Strathmann Biotec AG, Hamburg, Germany) in the presence or absence of 20 μM 15d-PGJ2 for 24 h. As a negative control, cells were cultured in a medium without TNF-α and 15d-PGJ2. For each cell culture set, expression of COX-2 was examined using Western blot analysis.

Western blot analysis

For Western blot analysis, cells were washed with PBS after the incubation period, scraped into 10 mM Tris-HCl, pH 7.4, 0.5 M NaCl, and sonicated three times for 30 s using Ultra S Homogenizer model (Taitec, Nagoya, Japan). After centrifugation at 10 000 g for 15 min at 4 °C, the supernatants were used for protein assay. The protein concentration was determined using Bradford method. The protein samples (50 μg) were run through 12.5% sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis. After electrophoretic transfer of the protein to nitrocellulose membrane, nonspecific binding was blocked by incubation with 3% gelatin in 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl (TBS) for 1 h at RT. After being washed three times with TBS containing 0.05% Tween 20 (TTBS), the blots were probed with a rabbit polyclonal IgG specific for human COX-2 (Immuno-Biological Laboratories, Gumma, Japan) or a PPARγ polyclonal antibody (Cayman Chemical, Ann Arbor, MI, USA) for 2 h. The blots were also probed with a monoclonal anti-β-actin antibody (SIGMA, St Louis, MO, USA) to be relatively quantified. β-Actin was used as a loading control. The membranes were then washed three times with TTBS and incubated for 1 h at RT with a biotinylated anti-rabbit immunoglobulin (Vector Laboratories, Burlingame, CA, USA) for COX-2 and PPARγ and a biotinylated anti-mouse immunoglobulin (Vector Laboratories, Burlingame, CA, USA) for (β-actin, respectively. After being washed three times with TTBS, the membrane was transferred to VECTASTAIN ABC Reagent (Vector Laboratories, Burlingame, CA, USA) and incubated in this solution for 30 min at RT. Diaminobenzidine was used as a substrate of peroxidase.

Statistical analysis

Statistical analysis was carried out by γ²-test or Fisher’s exact probability test. A result was deemed significant at P<0.05.

RESULTS

Detection of COX-2 and PPARγ in benign, borderline tumours and carcinoma

COX-2 and PPARγ were homogeneously stained in the cytoplasm of tumour cells in positive cases (Figure 1). The frequencies of COX-2 and PPARγ detection in ovarian tumours are demonstrated in Table 1. There was no significant difference in COX-2 positivity between samples of benign and borderline tumours (Table 1). The incidence of COX-2 detected in carcinomas was significantly higher than that detected in benign tumours (P<0.02) and higher with a marginal significance than that detected in borderline tumours (Table 1, P<0.058). On the other hand, the frequency of PPARγ detected in borderline tumours was significantly higher than that detected in benign tumours and carcinomas, respectively (P<0.0001, P<0.01, respectively).

COX-2 and PPARγ positivity in carcinomas was not correlated with clinical factors such as stage, histological type, lymph node metastasis and recurrence (Table 2).

Determination of COX-2 and PPARγ protein levels in ovarian tumour tissues

Levels of COX-2 and PPARγ proteins were determined by EIA in four benign tumours, three borderline tumours and 12 carcinomas. COX-2 and PPARγ proteins were not detected in the benign tumours at all (Table 3). The COX-2 protein levels of the three borderline tumours were below the measurement sensitivity, whereas PPARγ protein was detected in all of those ones (Table 3). COX-2 protein was detected in eight of the 12 carcinomas and the remaining four carcinomas lacked COX-2 protein (Table 3). In contrast, 10 of the 12 carcinomas lacked PPARγ protein and PPARγ protein was detected in only two carcinomas (Table 3). The level of PPARγ protein was below the measurement sensitivity in all of the eight carcinomas in which COX-2 protein was detected, whereas COX-2 protein lacked in the two carcinomas in which PPARγ protein was detected (Table 3).

\[\text{Figure 1} \quad \text{Immunohistochemical staining of COX-2 and PPARγ in ovarian carcinoma. Positive staining of COX-2 (A) and PPARγ (B) in ovarian tissues (scale bar, 50 μm). They are representative of all the positive samples. Tissues demonstrated in (A) and (B) are a serous adenocarcinoma.}\]

\[\text{Table 1} \quad \text{Detection of COX-2 and PPARγ in ovarian tumours}\]

| Tissue          | No. of cases | Case no. (%) | Case no. (%) |
|-----------------|--------------|--------------|--------------|
| Benign tumour   | 23           | 3 (13.0)     | 1 (4.5)      |
| Borderline tumour | 18           | 3 (16.7)     | 14 (77.8)    |
| Carcinoma       | 71           | 28 (39.4)    | 31 (43.7)    |

The incidence of COX-2 detected in carcinomas was significantly higher than that detected in benign tumours (P=0.002) and higher with a marginal significance than that detected in borderline tumours (P=0.058). The frequency of PPARγ detected in borderline tumours was significantly higher than that detected in benign tumours and carcinomas, respectively (P<0.0001, P<0.01, respectively).
Effect of 15d-PGJ$_2$ on COX-2 and PPAR$_y$ expression in cultured ovarian carcinoma cells

To examine whether COX-2 expression is controlled through PPAR$_y$ activation in ovarian carcinoma cells, OVCAR-3 cells and the ovarian carcinoma cells established at our hospital were cultured in a semiconfluent state in normal culture medium or in media supplemented with 0.1 – 20 μM 15d-PGJ$_2$, a PPAR$_y$ activator as described in Materials and Methods. Western blot analysis revealed that expression of PPAR$_y$ and COX-2 was increased and decreased, respectively according to 15d-PGJ$_2$ concentrations (Figure 2), suggesting that 15d-PGJ$_2$ activated PPAR$_y$ in both cell lines and PPAR$_y$ activation might result in suppression of COX-2 expression in the cells. Results shown in Figure 2 are representa-

tive of two separate experiments with two cell lines. β-Actin was used as a loading control.

Suppressive effect of 15d-PGJ$_2$ on cytokine-induced COX-2 expression in cultured ovarian carcinoma cells

TNF-α, an inflammatory cytokine, is known to increase COX-2 expression via nuclear factor NFκB pathway. To examine whether NFκB pathway is related to the signalling between PPAR$_y$ activation and COX-2 expression, OVCAR-3 cells were cultured in a semiconfluent state in normal culture medium or in media supplemented with TNF-α in the presence or absence of 20 μM 15d-PGJ$_2$ as described in Materials and Methods. Tumour necrosis factor-alpha induced COX-2 expression in OVCAR-3 cells (Figure 3). Addition of 15d-PGJ$_2$ to the media supplemented with TNF-α suppressed TNF-α-induced-COX-2 expression (Figure 3). β-Actin was used as a loading control.

DISCUSSION

The present study clearly demonstrated that the frequency and the degree of PPAR$_y$ expression were significantly increased as lesions progressed from a borderline tumour to carcinoma, and those of COX-2 expression were significantly increased as lesions progressed from a benign tumour to carcinoma, suggesting that low expression of PPAR$_y$ and high expression of COX-2 in precancerous lesions might be involved in progression of ovarian tumours to carcinoma. Independent studies have shown that induction of COX-2 and inactivation of PPAR$_y$ occurred during the development and progression of human breast carcinoma (Jiang et al, 2000; Ristimaki et al, 2002). On the other hand, inhibition of COX-2 and activation of PPAR$_y$ has been shown to prevent mammary carcinogenesis in experimental animals (Suh et al, 1999; Harris et al, 2000). Additionally, COX-2 selective inhibitors and PPAR$_y$ ligands could significantly attenuate the growth of human breast carcinoma cells (Elstner et al, 1998; Howe et al, 2001). Thus it has been suggested that high expression of PPAR$_y$ and low expression of COX-2 in the tumours might be involved in attenuating the capacity of the tumours to develop more malignant nature. Badawi and Badr (2003) described that an increase of COX-2 expression and a decrease of PPAR$_y$ expression in breast carcinoma tissues were paralleled by increases in the tissue levels of PGE$_2$ and decreases in 15d-PGJ$_2$ and their altered expressions might influence the development of human breast carcinoma.

COX-2 overexpression has been observed in many tumour types including gynaecological malignancies. Mechanistic study suggested that expression of COX-2 in uterine cervical carcinoma cells downregulated apoptotic processes and thus enhanced tumour invasion and metastasis (Affney et al, 2001). Li et al (2004) described that while COX-2 protein was not detected in normal
epithelium of the ovary, its protein was frequently expressed in ovarian epithelial carcinoma, suggesting that it might contribute to the carcinoma development or progression. Matsumoto et al (2001) found a significant correlation between expression of vascular endothelial growth factor (VEGF) and COX-2 in ovarian neoplasms and suggested that an increased expression of COX-2 might be associated with malignant transformation and tumorigenesis through the activation of VEGF. Denkert et al (2002) reported that expression of COX-2 was immunohistochemically detected in 42% of ovarian carcinomas and in 37% of borderline tumors, and described that COX-2 expression was an independent prognostic factor in human ovarian carcinoma. Although we found high frequency of COX-2 expression in ovarian carcinoma, we did not show the close relationship between expression of COX-2 and metastasis or recurrence in ovarian carcinoma. Large-scale prospective and retrospective studies are needed to clarify whether COX-2 expression is of practical utility as a prognostic factor.

PPARγ plays a crucial role in apoptosis and differentiation of a variety of cells. Induction of differentiation has been observed in several malignant cells as a result of stimulation by PPARγ (Mueller et al, 1998). PPARγ activation resulted in apoptosis of choriocarcinoma cells (Keelan et al, 1999), prostate carcinoma cells (Kubota et al, 1998), leukemic cells (Asou et al, 1999) and gastric carcinoma (Sato et al, 2000). More recently, Martelli et al (2002) reported that arrest of G1 cell cycle was observed and apoptosis was induced in thyroid carcinoma cells transfected with PPARγ. Breast carcinoma cells treated with PPARγ agonist show dramatic morphological changes, and express E-cadherin and b-casem, markers of breast cell differentiation (Elstner et al, 1998; Clay et al, 1999). Furthermore, it has been reported that differentiation and reversal of malignant changes were induced in CX-1 colon tumour cells treated with PPARγ agonist in Swiss nude mice (Sarraf et al, 1998). Zander et al (2002) described that activation of PPARγ transiently induced N-cadherin, the glioma differentiation marker, in human and rat glioma cells and in parallel, a subset of surviving cells showed de novo outgrowth of processes resembling astrocyte-like morphology based on PPARγ activation. Taking these results together including ours, activation of PPARγ and inhibition of COX-2 may be favourable for guiding neoplastic cells towards redifferentiation.

PPARγ signalling has been implicated in the control of COX-2 expression in certain tissues, although the exact mechanism that underlies PPARγ regulation of COX-2 expression remains to be elucidated. To date, there are several reports suggesting a reciprocal interaction between COX-2 expression and PPARγ activity (Inoue et al, 2000; Ikawa et al, 2001; Yang and Frucht, 2001). However, it is still unclear whether COX-2 expression is controlled through PPARγ signalling in ovarian carcinoma cells. We investigated whether PPARγ activity was involved in COX-2 regulation in human ovarian carcinoma cells, and found that 15d-PGJ2, a PPARγ ligand, reduced COX-2 expression in a dose-related manner, suggesting that COX-2 expression was regulated through PPARγ activity in ovarian carcinoma cells. The inhibitory effect of PPARγ activation on COX-2 expression is also reported in HT-29 human colon carcinoma cells (Yang and Frucht, 2001). Furthermore, it has been shown that NSAID suppress IL-1β-induced COX-2 expression (Xu et al, 1999), and inhibits TNF-α-induced COX-2 expression (Paik et al, 2000), suggesting that expression of COX-2 is under the control mechanisms by both cytokines and PPARγ systems. In fact, Inoue et al (2000) demonstrated that 15d-PGJ2 suppressed COX-2 promoter activity by interfering with the NFκB signalling pathway, and that transfection of a PPARγ expression vector into the endothelial cells suppresses the transcriptional regulation of COX-2 gene by 15d-PGJ2. Tumour necrosis factor-alpha is a strong inducer of COX-2 expression by stimulating the NFκB system (Yamamoto et al, 1995). As shown in Figure 3, the present result showed that COX-2 was induced by TNF-α-in ovarian carcinoma cells and that TNF-α-induced COX-2 expression was suppressed by 15d-PGJ2, suggesting that COX-2 expression was under the control of PPARγ and NFκB pathway in the ovarian carcinoma.

In conclusion, the reciprocal correlation between COX-2 and PPARγ found in the present study implicates that COX-2 and PPARγ may contribute to ovarian carcinoma induction. Thus, the present results suggested that ligand-mediated PPARγ activation suppressed COX-2 expression via the NFκB pathway in the ovarian carcinoma cells, and that high expression of PPARγ and low expression of COX-2 might play an important role in inhibiting ovarian carcinogenesis.

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